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13. ABSTRACT ***(Maximum 200 Words)***

The Bionetics Corporation operated and maintained a laboratory, which performed red blood cell preservation research for the Blood Research Detachment, Walter Reed Army Institute of Research at Building 503, Walter Reed Forest Glen Annex, Silver Spring, MD 20910. Contract staff completed one clinical trial and four *in vitro* protocols. The clinical trial was an evaluation of an investigational system for processing frozen red blood cells, which would extend the post-thaw shelf life from 1 to 15 days. The red cells processed by the system exceeded the required minimum of >75% survival 24 hours after autologous transfusion and < 1% hemolysis in the final product. The four *in vitro* protocols were designed to optimize the experimental additive solution storage system, expand our understanding of the mechanisms of action and test its utility for post-thaw storage of previously frozen red cells. Three manuscripts describing the results of research completed previously were published and a fourth is in press. The Bionetics Corporation advanced the mission of the Blood Research Detachment and the Walter Reed Army Institute of Research.

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FOREWORD

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N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Lloyd Lippert, Ph.D.

PI - Signature Date

Table of Contents

ANNUAL REPORT: DAMD94-C-4154

21 September 1999 - 20 September 2000

INTRODUCTION

Nature of the Problem:

Because combat is synonymous with bloodshed and blood replacement saves lives, the US Army Medical Research and Materiel Command maintains facilities and programs to develop improved blood products.

The Background of the Previous Work:

The US Army has, for decades, conducted research in red blood cell preservation and the production of acellular hemoglobin solutions for use in combat casualty care. From 1974 through 1992, that research took place at the Letterman Army Institute of Research (LAIR) located at the Presidio. The LAIR facility was closed as the result of Base Realignment and Closure actions and the Blood Research Detachment was relocated to leased laboratory space at 1413 Research Boulevard, Rockville, Maryland. On 19 September 1994 The Bionetics Corporation (TBC) was awarded a contract to operate and maintain equipment and provide technical support to the Blood Research Detachment (BRD). For the first two years of the contract, the contractors supported the Hemoglobin Production Facility (HPF) and integral analytical chemistry laboratory, which provided quality control and characterization testing. The HPF produced several hundred liters of a high-purity hemoglobin-based blood substitute material for research. The acellular hemoglobin solutions manufactured by the HPF were based on biochemical modification of stroma-free hemoglobin as described in the literature. [1,2,3,4] A detailed description of the hemoglobin solution production process and process improvements is contained in a manuscript, published in October 1997 by the journal "Biologics", the journal of the International Association of Biological Standardization. A reprint copy of the complete journal article, Highsmith FA, Driscoll CM, Chung BC, Chavez MD, Macdonald VM, Manning JM, Lippert LE, Berger RL, and Hess JR. "An Improved Process for the Production of Sterile Modified Haemoglobin Solutions" Biologics,

1997;25:257-268, was included in a previous annual report. During the last several months of HPF operation, the contract staff also supported the US Navy lipisome encapsulated hemoglobin research.[5,6] HPF related activities were terminated on 20 September 1996 and the facility closed. Since then, the production facility equipment was either turned-in for disposal or transferred to other Institute activities for their use.

Contract staff has continuously operated and maintained the blood cell preservation research of the Blood Storage Laboratory (BSL), a fully equipped red cell research laboratory currently staffed by 3 full-time and 2 part-time employees. One of the Blood Banking Specialist members of this team is also the Project Manager of the contract and the contract designated Principle Investigator holds the specialty certification in Blood Banking. A technologist who also holds the same specialty certification is also a part of the contract staff. A list of contract staff as of the close of the FY is included at Appendix 1.

The current Food and Drug Administration (FDA) licensed anticoagulant, preservative solutions allow storage of red blood cells at 4°C for 42 days after collection. Work by Meryman *et. al.*[7,8] and Greenwalt, Dumaswala and colleagues [9,10] indicate potential for extended storage. Greenwalt and Hess have developed an experimental additive solution which preserves red cells for 63 days with average red cell survival of at least 75%.[11] Subsequent modifications have yielded greater than 75% 24-hour post transfusion survival after 10 week of storage.[12]

It is estimated that one half million units of blood expire per year in the United States. It is further estimated that if expiration were extended to 8 weeks, one-third of the expired units now discarded would be transfused.[13] Extended shelf-life of liquid stored blood would have significant utility to the Armed Services Blood Program as it supports the Department of Defense blood transfusion requirements world-wide. The major advantages of an eight-week extended shelf life red cell product include, reducing resupply requirements by up to 30%, reducing the blood collection requirements to replace outdated products, and increasing the practicality of rotating aged but unexpired red cell products from the theater of operations to continental U.S. (CONUS) treatment facilities where they are more likely to be used. Extending the storage time to 12 weeks would halve the resource requirements. The net result is reduced costs through enhanced utilization of a scarce, perishable resource.

The laboratory has expanded its research from the preservation of red cells stored exclusively at refrigerator temperatures (2 - 6°C) to the preservation of red cells after frozen storage. Red cells destined for frozen storage are initially refrigerated for a few days, ordinarily up to 6 days, before they are mixed with the cryoprotectant glycerol and then stored in a freezer -65°C or colder for up to 10 years. Before those red cells can be transfused, they must be thawed and glycerol removed. Currently available systems for the processing of frozen blood limit the post thaw storage to 24 hours per FDA mandate. The 24-hour limit is imposed because the current systems are open, as defined by the FDA, and therefore vulnerable to bacterial contamination. Storage beyond 24-hours would allow the proliferation of the contaminating bacteria to levels, which would harm the recipient. Under development are two systems, which would allow post thaw storage beyond 24-hours because the fluid paths are closed to potential bacterial contamination. Therefore the limitation on the post thaw storage is limited by the survival of the cells after transfusion. Candidate systems are designed to allow two weeks of post thaw storage, an enhancement, which would greatly expand the utility of frozen red cells for the military civilian uses.

The Purpose of the Present Work:

The BSL evaluates the effectiveness of candidate red blood cell anticoagulant preservative systems and storage strategies along with their potential for further development. The BRD supports the Military Blood Program by providing data which will evaluate the safety, effectiveness, and practicality of new products or procedures related to the collection, processing, safety and distribution of red blood cells. Prior to the formulation of any of the clinical trails involving novel storage solutions, a series of *in vitro* protocols are initiated to gain insights into the biochemical mechanisms responsible for energy production and maintenance of red cell membrane integrity.

Organization of Report:

During this reporting period, the BSL has performed research under one clinical trial protocol, a phlebotomy type protocol which permits collection of blood from volunteers for *in vitro* research and three *in vitro* research projects. The initiation of another clinical trial awaits

approval by the Human Subjects Research Review Board the availability of the instrument and instrument specific disposables being evaluated.

The BACKGROUND, METHODS, RESULTS, and DISCUSSION / CONCLUSION from each research effort will be described separately in order to maximize continuity. A short description of the status of each project begins the description of each protocol. If a manuscript has either been submitted for publication or published, the abstract from the manuscript is reproduced in the body of the report and the entire manuscript or copy of the published journal article is added to the report as an appendix.

The phlebotomy type protocol activities will be described first, followed by descriptions of the clinical trial. Also included are descriptions of clinical trials, completed in previous fiscal years, but for which results were published in the current period. The ultimate goal of the clinical trial is to determine if the mean 24 hour post-transfusion red cell survival at the end of the storage period exceeds 75% in a minimum of ten volunteers. Furthermore, at least 99% of the cells collected must also remain intact on the final day of storage. This consensus standard has been adopted by the FDA. The report is concluded by descriptions of a series of *in vitro* protocols listed in order they were initiated.

Description of research conducted:

A. "Phlebotomy Procedures for Use on Human Subjects" WRAIR #776, HURRAD Log #A-6664.

BACKGROUND

Aspects of red blood cell physiology critical to blood storage are species specific; therefore valid *in vitro* studies of the red blood cell storage lesion require freshly collected human blood. The quantities required range from as little as 3.0 mL to as much as a full unit, 450 mL.

METHODS

Volunteers are recruited from within the Detachment, other tenants of Building 503, Forest Glen, Walter Reed Army Medical Center (WRAMC) and the immediately surrounding community. Potential volunteers were informed fully as to the risks of donation and screened for

anemia, transfusion transmitted diseases (TTD) and medical conditions, would make blood donation unsafe using the criteria of the American Association of Blood Banks[14] and the Food and Drug Administration 21CFR640.[15] The total amount of blood collected in an 8 week period is limited to 525 mL. A physician, certified in Advance Cardiac Life Support, was present at all full unit phlebotomies. Volunteers are compensated for their blood donations IAW 24 USC 30 and AR 40-2. A single modification to the protocol was approved to allow blood collection from 20 or more volunteers specifically for the biochemical defense program. No collections have been made in support of this protocol modification as of 30 September 2000.

A personal computer database is used to maintain documentation of all volunteer related transactions and assure compliance with donation volume and interval limitations. Trained contract staff and selected, trained active duty personnel performed the phlebotomies.

RESULTS:

During fiscal year 2000, the following blood collections were made as described in the following table.

Table 1: Blood Collections for FY 2000

Volume Collected	Blood Storage Laboratory (BSL)	BSL Clinical Trial	Other Blood Research	Total
1 - 50 mL	56	N/A	47	105
51 - 100 mL	4	N/A	23	27
101-200 mL	1	N/A	11	12
>200 mL	0	N/A	0	0
Units	97	11	9	117
		Total Phlebotomies Performed		261

The column labeled Blood Storage Laboratory (BSL) accounts for the collections for *in vitro* protocols, instrument standardization, test development and volunteer screening. The eleven unit collections under the column "BSL Clinical Trial" were used for red cell survival studies for the evaluation of the Haemonetics MDL 215. A complete description of that protocol follows. "Other Blood Research" collections were made to support of other Blood Research Detachment investigators.

Fifty-seven new volunteers were enrolled during the fiscal year. Forty-two of the enrolled volunteers were converted to inactive status because they had left the area and were otherwise unavailable or withdrew for unstated personal reasons; three additional volunteers were permanently deferred because of a disqualifying conditions. One hundred fifty-four sets of transfusion-transmitted disease screening tests were performed during the report period. Four reactive screening results were reported. They were two separate reactive results for the antibodies to the human immunodeficiency viruses (HIV) I/II, and single reactive results for antibodies to the hepatitis C core antibody (HBc) and the human T-cell lymphotrophic viruses 1 and 2 (HTLV1/2). Only the anti-HBc result confirmed on subsequent testing. The anti-HIV I/II and anti-HTLV1/2 positive screen results were not confirmed with western blot analysis. The two anti- HIV I/II results were from separate specimens from the same volunteer, who had previously received an experimental HIV vaccine. The two remaining reactive results were from two additional volunteers. The volunteers were informed of the test and counseled by a physician.

A single episode of donor dizziness, tachycardia and hematoma at the phlebotomy site occurred during the 257 phlebotomies. Such events occur occasionally during blood donation and are not unusual. The staff employed the procedures specified in the protocol to deal with donor reactions; there were no sequelae.

DISCUSSION / CONCLUSION

One hundred percent of collections were preformed in support of BRD related research. In the previous fiscal year, seven percent of collections were performed for the Transfusion Medicine Research Laboratory of the Naval Medical Research Institute and the Armed Forces Institute of Pathology with whom we were co-located while at the Gillette Building. The blood collection requirements of the BRD were safely provided from a pool of healthy, screened volunteers.

B. "Evaluation of *in vitro* Storage of Human Red Blood Cells after Sterile Frozen Storage, Processing on the Haemonetics 215 and Two Weeks of Liquid Storage in AS-3" WRAIR # 714.

and

C. "Evaluation of *in vivo* viability of human red blood cells after sterile frozen storage and processing on the Haemonetics 215 and 2-week liquid storage in AS-3". WRAIR #715, Log # A-8654.

STATUS:

These two protocols were completed and fulfilled our obligations, which are part of a Memorandum of Understanding between the Office of Naval Research and WRAIR. WRAIR was but a part of a multi-center study to provide data for the eventual licensure of this experimental system. Four other sites are performing *in vitro* evaluations similar to the protocol #714 and one other site is performing red cell survival studies on Haemonetics 215 processed red cells, similar to protocol #715. All testing except the 24-hour post-transfusion red cell survival measurements, however was performed at the Naval Blood Research Laboratory (NBRL) in Boston, MA. Locally, volunteers were recruited, blood drawn and processed with the Haemonetics instrument, stored either in the frozen or liquid state for the prescribed time, and samples collected for shipment to NBRL. A manuscript describing the results of the evaluation from all participating sites was submitted for publication in the journal TRANSFUSION.

The Haemonetics Corporation has prepared and submitted a 510K application for Food and Drug Administration clearance for use. Approval is pending.

ABSTRACT

The FDA has approved the storage of frozen red blood cells (RBC) at –80°C for 10 years. Following deglycerolization the RBC can be stored at 4°C for no more than 24-hours because open systems are currently being used in the glycerolizing and washing procedures, introducing the potential for bacteriologic contamination. Our laboratory has been evaluating the Haemonetics Mode 215, an automated functionally closed system, used for both glycerolization

and deglycerolization processes. We report here on a multi-center study to assess the quality and sterility of RBC processed using this instrument. Studies were performed at three military sites and two civilian sites, each performing in vitro testing of approximately 20 units of RBC. Additionally, at one military site and at two civilian sites, in vivo studies were conducted. At each site, about ten units of RBC that had been stored at 4°C in AS-3 for 15 days after deglycerolization were auto-transfused. At one of the civilian sites, NBRL, each of ten volunteers was auto-transfused on two occasions in a randomized manner, one with previously frozen RBC that had been stored at 4°C in AS-3 for 15 days after deglycerolization and once with liquid-preserved RBC that had been stored at 4°C in AS-1 for 42 days. Measurements were made of in vitro RBC recovery, hemolysis and bacteriologic cultures. Twenty-four-hour post-transfusion survival values were assessed by double label procedures and the 51cr 1-1/2 values were measured. For 142 units studied at the five different sites, the mean in vitro freeze-thaw-wash (FTW) recovery value was 87 percent ± 5 (SD). The mean supernatant osmolality on the day of deglycerolization was 297 mOsm/kg H_2O ± 5 (SD), and the mean percent hemolysis after storage at 4°C in AS-3 for 15 days was 0.60 percent ± 0.2 (SD). At three sites, at which 24-hour post-transfusion survival values were measured by three different double label procedures, a mean 24-hour post-transfusion survival of 77 percent ± 9 (SD) was observed for a total of 35 auto-transfusions in 12 females and 24 males of previously frozen RBC that had been stored at 4°C in AS-3 for 15 days following deglycerolization. The multi-center study demonstrated the acceptable quality of RBC glycerolized and deglycerolized in the automated Haemonetics Model 215 instrument.

BACKGROUND / METHODS / RESULTS / DISCUSSION / CONCLUSION

A copy of the submitted manuscript describing the background, methods, results, discussion and conclusion from these two clinical trials is attached to this report as Appendix 2.

D. "Evaluation of *in vivo* viability of Human Red Blood Cells After Sterile Storage, Processing on the Mission Medical FBPS and 2 Weeks of Liquid Storage in AS-3" WRAIR # 817, HSRRB Log #A - 10159.

STATUS

The Frozen Blood Processing System (FBPS) has been developed by Mission Medical Incorporated (MMI) under an MRMC SBIR initiative. Prototype instruments and disposables have been manufactured and tested extensively at MMI, onboard US Navy vessels and at the Institute. The FBPS is designed to perform the same essential functions as the Haemonetics Model 215 (MDL 215) evaluated previously and described in a preceding section of this report. The instruments for the evaluation have been produced and the production of the disposable sets necessary to perform the function is pending. Though the system is not yet available for evaluation, because the time to approval for clinical trial protocol may require 3 - 6 months, the protocol approval process was initiated in advance. The protocol has received all Institute approvals, approval by the Walter Reed Army Medical Center Radiation Control Committee and is pending final approval by the HSRRB.

BACKGROUND

The preparation of a unit of blood for frozen storage and eventual transfusion involves a series of related processes. Red cells destined for frozen storage are initially refrigerated for a few days, ordinarily up to 6 days, before they are mixed with the cryoprotectant glycerol and then stored in a freezer -65°C or colder for up to 10 years. Before those red cells can be transfused, they must be thawed and glycerol removed. Currently available systems for the processing of frozen blood limit the post thaw storage to 24-hours. The 24-hour limit is imposed because the current systems are open as defined by the FDA and therefore vulnerable to bacterial contamination. Storage beyond 24-hours would allow the proliferation of the contaminating bacteria to levels, which would harm the recipient. The FBPS would allow post thaw storage beyond 24-hours because the fluid paths are closed to potential bacterial contamination. Therefore the limitation on the post thaw storage is limited by the survival of the cells after transfusion. Hess and colleagues have demonstrated successful three-week storage followed by greater than 75% survival of the

red cells 24-hours after transfusion into autologous recipients. Two weeks of post thaw storage was chosen for the evaluation of the FBPS, which is the same end point for the MDL 215 evaluation.

However, the FBPS differs from the MDL 215 in two significant ways. First, the FBPS provides a higher level of automation, which should serve to allow the operation of more instruments, thereby extending the ability of a technician to process more units of blood faster when needed. The FBPS also utilizes a different technology to remove the glycerol. The MDL 215 utilizes centrifugal separation of the liquid glycerol and the red cells for glycerol removal. The FBPS utilizes dialysis by incorporating hollow fiber filters similar to those used for renal hemodialysis. The hollow fiber filters retain the red cells; the glycerol and wash solution are discarded in the filter effluent. The primary advantage of the dialysis-based separation is the elimination of the internal motor required to drive the centrifuge required for centrifugal separation. The enhanced level of automation, simplicity of design combined with two weeks of shelf life for the processed blood would greatly expand the utility of frozen red cells for the military and civilian uses.

METHODS

We will recruit approximately 15 volunteers (permission to enroll up to 20 is requested) with the expectation of getting a minimum of 10 of them through the protocol. Each volunteer's participation will last a minimum of ten weeks in order to complete two red cell survival studies. Recruited volunteers will be screened and, if acceptable, will donate a full unit of blood as described in WRAIR Protocol #776, "The Repeated Use of Small Volumes of Purchased Human Blood in Laboratory Tests, Instrument Calibration and In Vitro Experiments." The blood will be glycerolized using the automated procedure on the Mission Medical FBPS. The glycerolized blood will be frozen at -65C or colder for at least a month. Two weeks before the scheduled day of reinfusion the unit will be thawed and deglycerolized using the automated procedure on the Mission Medical FBPS. The cells will then be stored at 4°C for 14 days in a conventional blood bank refrigerator. On the fifteenth day of liquid storage, the stored cells along with 6 mL of fresh cells drawn from the same volunteer will be radiolabeled and reinfused to measure the viability of

the stored cells as described in WRAIR Protocol # 554, "Type protocol for measuring the recovery and survival of autologous human erythrocytes *in vivo*."

Blood units will be stored as above before *in vivo* survival testing. Three to five days before a reinfusion, the blood will be inspected and cultured for bacterial contamination. On the day of the reinfusion, the bacterial cultures will be confirmed to be negative before the unit is gently mixed and the post-storage sample taken for *in vitro* testing and radioisotope labeling. The *in vivo* survival testing of the stored red blood cells will be done as per the approved procedure, WRAIR Type Protocol #554. This procedure has two basic parts, which are performed simultaneously. In the first part, fresh red cells are radiolabeled with ^{99m}Tc, which have a half-life of only 6 hours. These are injected followed by rapid, timed sampling and radiation counting to accurately measure the blood volume. Gamma emissions from ^{99m}Tc labeled cells are essentially undetectable 24-hours after injection. In the second part, autologous stored red cells are radiolabeled with ^{51}Cr,which has a half-life of 27.7 days. The ^{51}Cr labeled red cells are injected and their survival is tracked for two weeks by analyzing timed blood samples for gamma emissions.[16] The mean 24-hour red cell survival for all the volunteers must exceed 75% for the storage system to be considered effective by the FDA. The FDA also requires the red cell survival studies be repeated at another laboratory with another set of volunteers. Therefore, the same evaluation will be conducted at another laboratory chosen by MMI. The results will be combined for a 510k submission and eventual clearance for clinical use by the FDA.

The clinical trial will be preceded by an *in vitro* evaluation involving 40 units of blood. The *in vitro* phase of the evaluation is designed to assess the quality of the red cells and the degree to which red cell quality might be affected by the use of leukoreduction (LR) filtration immediately following collection of the blood. Red cell quality will be assessed with an array of biochemical and hematological testing. The more important parameters being assessed are maintenance of red cell ATP and level of hemolysis. The effect of LR will be measured by comparing the results of the biochemical and hematological testing when blood is collected with and without filtration. Twenty units each will be collected into AS-3 and AS-1 blood collection systems. Half of each of the AS-3 and AS-1 groups will be filtered; the remainder will not be filtered. Based on previous research from our laboratory, hemolysis is expected to be lower in the filtered cells.[17] The effect of the differing collection systems is unknown.

It is important to evaluate the effect of leukoreduction because current practice is mixed. Furthermore, white blood cells and cell fragments are retained during processing with the FBPS; whereas, white blood cells are removed with the supernatant during processing with the centrifugal based systems. If as expected the LR product will have lower hemolysis, it is important to have data determine if processing with the FBPS is sufficiently robust.

Two collection systems are being used because, again, current practices are mixed. Approximately one half of the blood collected in the USA is collected into AS-1. The next most used system is AS-3. The second aspect of the collection system issue involves the uses of AS-3 in the post thaw storage stage of the process. Because the pre-freezing stage of the process is dissociated for the post thaw stage, it is important to address the question regarding the affect of pre-freeze storage solution on the post thaw quality. If as expected there is no significant effect for the choice of collection system, the FBPS would have greater utility.

E. "Evaluation of the *in vivo* Viability of Red Blood Cells Stored for Prolonged Period in the Leukotrap® RC AS-24 System" WRAIR #572, HURRAD Log #A-6986.

and

F. "Evaluation of the *in vivo* Viability of Red Blood Cells Stored for Eight Weeks in the Leukotrap® Whole Blood AS-3 System" WRAIR #591, HURRAD Log #A-7089.

STATUS:

WRAIR protocols # 572 and # 591 were performed under terms of a cooperative research and development agreement (CRDA) with the MEDSEP Division of the PALL Filter Corporation. Both protocols were completed in a previous fiscal year and individually described in a previous annual report. WRAIR protocol #572 evaluated an experimental system incorporating a new formulation of chemicals already used in FDA approved solutions for blood storage, and an integral white blood cell removal filter; the system is designated AS-24. The objective of the first protocol was to determine if this unique preservative formulation coupled with pre-storage white cell removal would permit storage for eight weeks. WRAIR protocol

#591 was designed to evaluate a second experimental system for potential eight-week red cell storage. A secondary purpose was to evaluate effects of periodic mixing combined with either horizontal or vertical storage. The blood collection system utilized in protocol # 591 differed from the system employed in protocol # 572 in two aspects. First, the FDA licensed AS-3 replaced the experimental hypotonic AS-24 preservative solution in the test units. Second, the system was reconfigured to accomplish white cell removal during collection of the whole blood rather than after the preparation of the packed cells, as was the case in protocol #572. The results from both protocols were combined into a single manuscript, which was published in the journal TRANSFUSION, August 2000.

ABSTRACT

Hypotonic storage solutions and WBC filtration are both reported to improve RBC viability. This study tested the ability of an investigational hypotonic storage solution (AS-24, MEDSEP Corp.) to extend the viability of liquid-stored RBCs to 8 weeks. In a pair of cross-over trials, 11 RBC units, WBC-reduced by filtration and stored in AS-3 for 8 weeks, were compared with units from the same donors that were stored for 6 weeks in AS-3. In the second comparison, 13 RBC units, WBC-reduced by filtration and stored in AS-3 for 8 weeks, were compared with units from the same donors that were stored for 6 weeks in AS-3. Viability was measured by the $^{51}Cr/^{99m}Tc$ double isotope method. RBC viability at 8 weeks averaged 64±3 percent in the AS-24 units and 67±2 percent in the AS-3 units. It was equal at 77±3 percent and 77±2 percent after 6 weeks' storage in As-3 in both trials. Pre-storage WBC reduction and storage in AS-24 did not extend RBC viability to 8 weeks. The improved viability previously demonstrated with storage of dilute suspensions of RBCs in hypotonic solutions is probably caused by factors other then the hypotonicity.

BACKGROUND / METHODS / RESULTS / DISCUSSION / CONCLUSION

A copy of the published manuscript describing the background, methods, results, discussion and conclusion from these two clinical trials is attached to this report as Appendix 3.

G. "Evaluation of the in vivo viability of red blood cells after 8- and 9 -week storage in Terumo's EAS-61 Additive solution" WRAIR #713, Log #A-8655.

STATUS:

WRAIR #713 was closed because the corporate partner, Terumo Corporation, was not responsive to requests to produce the materials needed for this research. The protocol outlined our plans to test an experimental solution in a minimum of ten volunteers after both eight weeks and nine weeks of storage in a random double crossover clinical trial using AS-5 as the control storage system. The EAS-61 additive solution is an investigational product developed at another institution under an extramural contract and to have been manufactured under license to the Terumo Corporation. Survival of red cells stored in EAS-61 for nine weeks has exceeded the 75% minimum by a clear margin in limited testing performed in the contractor's laboratory. The data collected in this protocol would have been used to support FDA licensure. No apparent progress was made in producing the materials nor were there any reasonable prospects for such in the foreseeable future; therefore, the protocol was closed.

H. "The *in vitro* storage characteristics of red blood cells stored in increasing volumes of AS-1"

and

I. "The *in vitro* storage characteristics of red blood cells stored in Experiment Additive Solutions EAS-61 and EAS-64"

STATUS

Both the *in vitro* study of the additive solution AS-1 and the experimental additive solution EAS-61 were completed in previous fiscal years. The description and results of these two studies and a third study involving both EAS-61 and EAS-64, conducted by a collaborator, Dr. Tibor Greenwalt from the University of Cincinnati and Hoxworth Blood Center, were combined in a single manuscript. However, it was not until August 2000 the results were published in the journal TRANSFUSION.

All three protocols employed the same experimental design in which either three or four individual blood unit blood donations were pooled and mixed. The pooled, mixed blood was divided into either three or four aliquots, the number determined by the number of units pooled. Each aliquot receiving a different treatment. The treatments differed in either the composition or amount of the additive solution. Because pooling moderates or averages the sometimes significant observed differences in storage characteristics of blood from different volunteers, far more powerful data can be obtained than when each individual's blood unit is analyzed separately. The data from these experiments, when analyzed together, provided insight into the relative contributions of additive solution volume and additive the supplements mannitol and phosphate.

ABSTRACT

RBC ATP concentrations are the most important correlate of RBC viability. Tests were performed to determine whether increased AS volume, pH, and phosphate content increased stored RBC ATP concentrations. In three studies, packed RBCs were pooled in groups of 3 or 4 units and realiqouted as combined units to reduce intra-donor differences. Pooled Units were stored in the licensed additive solutions, AS-1 or AS-5, which contain saline, adenine, glucose and mannitol (SAGM), or in experimental additive solutions (EAS) containing SAGM and disodium phosphate. Ten pools were stored in AS-1 at RBC concentrations equivalent to 100, 200, or 300 mL of AS. Six pools were stored in 100, 200, 300, or 400 mL volumes of EAS-61.Ten pools were stored in 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64. RBC ATP concentration and other measures of RBC metabolism and function were measured weekly. RBC ATP concentrations decreased sooner with storage in increasing volumes of AS-1. In EAS-61 and EAS-64, RBC ATP concentrations initially increased and stayed elevated longer with increasing AS volume. The addition of disodium phosphate to SAGM AS increases the RBC ATP concentrations. Reducing storage Hct appears to have a separate beneficial effect in reducing hemolysis.

BACKGROUND / METHODS / RESULTS / DISCUSSION / CONCLUSION

A copy of the published manuscript describing the background, methods, results, discussion and conclusion from these two clinical trials is attached to this report as Appendix 4.

J. *In vitro* comparison of Red Blood Cells Stored in EAS-61 in Polyolefin versus Polyvinyl Chloride Storage Bags.

STATUS:

This protocol was completed during the current FY and a manuscript submitted for publication to the journal TRANSFUSION and rejected because a major premise for the research had not yet been published in the peer-reviewed literature. The research was constructed around the premise that the know deleterious effects of storing red cells in non-diethylhexylpthalate (DEHP) plasticized polyvinyl chloride plastic could in part or *in toto* be overcome or overridden by use of EAS-61. It was not until August of 2000 the data supporting the superiority of EAS-61 and a variant, EAS-64, was published.[11,12]

ABSTRACT:

Packed red blood cells (PRBCs) must be stored in polyvinyl chloride (PVC) bags plasticized with di-2-ethylhexyl phthalate to achieve their full storage life with conventional storage solutions. Better additive solutions may remove this requirement. Two hundred mL of Experimental Additive Solution - 61 (EAS-61) maintains PRBCs for 9 weeks. Twenty-four units of PRBCs were pooled in groups of 4 units. Each pool was realiquoted into 4 units, and stored, six pooled units per arm, in 1) 100 mL of EAS-61 in PVC, 2) 200 Ml of EAS-61 in PVC, 3) 100 mL of EAS-61 in polyolefin (PO), and 4) 200 mL of EAS-61 in PO. Hemolysis, RBC morphology indices, RBC ATP concentrations, and other measures of RBC metabolism and function were measured weekly. RBC hemolysis exceeded 1% by 3 and 5 weeks in PO bags containing 100 and 200 mL of EAS-61. In PVC bags, hemolysis reached 1% at 8 and 10 weeks. RBC ATP concentrations were 1μ

BACKGROUND: The impetus for this study was threefold. First, the polyvinyl chloride (PVC) plastic used in blood collection system bags, as well as most of the IV solution bags and dialysis equipment, retains its pliability through the use of a plasticizer diethylhexylpthalate (DEHP). DEHP is a known health hazard in laboratory animals and a perceived health risk in humans. Second, the manufacture of PVC plastic requires the use of vinyl chloride, a carcinogen. Third, the incineration of PVC, the customary method of medical waste disposal, produces a variable amount of dioxins, another health and environmental hazard. For these reasons, it has been proposed to replace PVC with other non-PVC plastics. There is some evidence in the literature, however, that DEHP has a beneficial effect on red cell storage,[18,19,20,21,22] the storage lesion, i.e., the deterioration of the red cells as measured by loss of ATP, disk to sphere morphological changes and hemolysis. Hemolysis is accelerated when red cells are stored in non-PVC containers. This study was designed to determine the extent of the detriment created by storage in polyolefin plastic (PO), a non-PVC plastic, and to determine if the superior red cell preservation qualities of the experimental additive solution EAS-61 was sufficient to overcome the deficit created by depriving the red cells of exposure to DEHP during storage.

METHODS, RESULTS, DISCUSSION AND CONCLUSION:

A copy of the submitted manuscript describing the methods, results, discussion and conclusion for this research is attached to this report as Appendix 5.

K. "Comparison of EAS-61 and AS-3 RBC Stored in either 100 mL or 200mL of Additive Solution: Liquid Storage"

and

L. "Comparison of EAS-61 and AS-3 RBC Stored in either 100 mL or 200 mL of Additive Solution: Frozen Storage"

STATUS:

This pair of linked minimal risk *in vitro* protocols was initiated and completed during the FY 2000.

BACKGROUND:

This pair of linked protocols was designed to answer some research questions which arose after analyzing the results of previous work in our laboratory with the experimental EAS-61 and the licensed AS-3 additive solutions both in liquid stored and previously frozen red cells. The previous work gave indications of differences in performance characteristics of the two solutions and the differences were perhaps volume dependent. However, this was the first time AS-3 and EAS-61 were compared directly and using both 100 and 200 mL volumes per unit of red cells. It was postulated, the differences indicated by the previous work would become apparent when multiple units of identical ABO group were pooled and then split into the different treatment groups. We were particularly interested in gathering data on the use of EAS-61, as a substitute for AS-3, for the post thaw storage of previously frozen red cells. Furthermore, Yoshida and colleagues had recently reported on the use of AS-3 under oxygen depleted conditions.[23] This raised the question, "Would substitution of EAS-61 in either 100 or 200 mL volumes under ambient condition of storage achieve comparable results to 100 mL of AS-3 under oxygen depleted conditions?"

METHODS:

Volunteers were recruited under the provisions of WRAIR protocol #776, "The Repeated Use of Small Volumes of Purchased Human Blood in Laboratory Tests, Instrument Calibration and In Vitro Experiments." Each volunteer donated one unit. Forty-eight units were collected. The 48 units were divided into two sets of 24 units of blood, one set for the "liquid" protocol and the second for the "frozen" protocol. In each set, four units of identical ABO group were pooled and after mixing, the blood was divided into 4 aliquots. The first set of 24 aliquots, the "liquid" protocol, were stored at 4^0C after the addition of 100mL of EAS-61 to one aliquot, 200 mL of EAS-61 to the second, 100 mL AS-3 to the third and 200 mL AS-3 to the fourth. Each of the aliquots was sampled on the day of preparation and weekly thereafter for 9 weeks. Samples were analyzed for CBC, blood gas analysis (BGA), centrifuged hematocrit, red cell morphology, supernatant hemoglobin, sodium, potassium, and phosphate, plus whole blood ATP, glucose and lactate.

The second set of 24 units was used in the "frozen" protocol. The 24 units were also pooled in groups of four of identical ABO group and divided into four aliquots as in the "liquid" protocol. At this point the aliquots were individually prepared for frozen storage using the closed system Haemonetics MDL 215. The glycerolized cells were stored a minimum of 4 weeks at -65°C or colder and deglycerolized, again using the Haemonetics instrument. As the final step of preparing the RBC for 4°C storage, the same volumes of EAS-61 and AS-3 were added to the aliquots as in the "liquid" protocol. Each aliquot was sampled after addition of the additive solutions and weekly thereafter during 5 weeks of 4°C storage. Testing was identical to that of the "liquid" protocol.

Statistical comparison of ATP concentrations and hemolysis of RBC stored in 100 mL or 200 mL of EAS-61 with RBC stored in the same volumes of AS-3 was the primary outcome analyzed. Secondarily, a statistical comparison of additional hematologic and biochemical parameters of RBC stored in 100 mL or 200 mL of EAS-61 with RBC stored in the same volumes of AS-3. The data from the "liquid" and "frozen" studies were analyzed separately. Analysis of variance was performed using the statistical functions available in MS Excel®. Differences with a probability of $p \leq 0.05$ were considered significant.

RESULTS:

Significant results from this study were as follows. Summary data tables and graphs are located at Appendix 6.

ATP/Frozen: ATP was better maintained with EAS-61 and remained at higher levels through 35 days of storage, i.e., approximately three micro-moles /g HGB in EAS-61 versus approximately one μmol/g HGB when stored in AS-3. A graphic description of the data follows

ATP/Liquid: Though the differences were not significant, the initial increases in ATP concentrations reached higher levels and were sustained longer in both volumes of EAS-61 compared to the AS-3 stored cells. However, after nine weeks of storage the concentrations were nearly identical, approximately 2.25 μmol/g Hgb for all but the cell stored in 200 mL of EAS-61 where the ATP levels averaged 2.95 μmol/g Hgb. The data is data is contained in a graph, which follows.

Hemolysis/Frozen: Hemolysis in EAS-61 at two weeks of post deglycerolized storage was 0.96 % (SEM = 0.09) and 1.03% (SEM = 0.29) for 100 mL and 200 mL storage respectively. Hemolysis in both volumes of EAS-61 exceeded 2% at 35 days of storage. Hemolysis in AS-3 stored cells was 0.63% (SEM = 0.01) and 0.2% (SEM = 0.02) when stored in 100 mL and 200 mL respectively for 14 days and did not exceed 1% until 28 days of storage in 100 mL and 35 days of storage in 200 mL of additive solution.

Hemolysis/Liquid: Graphically, the hemolysis results formed three different curves. Hemolysis increased more rapidly and reached far higher levels, 2 -3 times the others, when 100 mL of AS-3 was used as the storage solution. Between 42 and 49 days of storage, the hemolysis exceeded 1.0%. Cells stored in either 200 mL of AS-3 or 100 mL of EAS-61 gave highly similar results with hemolysis exceeding 1.0% between 56 and 63 days of storage. The cells stored in 200 mL of EAS-61 were the most resistant to hemolysis, with levels averaging 0.70 % after 9 weeks of storage.

RBC Morphology/Frozen: The EAS-61, in both volumes, maintained the morphological index at higher levels compared to the AS-3. As storage time progressed the differences increased.

RBC Morphology/Liquid: The pattern of red cell morphological index scores paralleled the scores from the frozen storage data when examined graphically with one variation. Storing previously frozen red cells with either 100 or 200 mL of EAS-61 produced nearly identical data. Whereas, liquid storage with 100 mL EAS-61 produced data intermediate between 200 mL of EAS-61 and either volume of AS-3.

Other Parameters/ Liquid and Frozen: The results from the other parameters analyzed were unremarkable.

DISCUSSION:

Neither solution, AS-3 or EAS-61 provided the almost universally observed boost in ATP concentrations one to two weeks after beginning of storage when adenine supplemented solutions are used in storage of frozen red cells. The most likely explanation for the absence of the ATP surge is a lowering of the pH during the initial 5 days of refrigerated storage before freezing and later during the deglycerolization wash process. Certainly, the pH of the stored blood would be

expected to fall during 5 days of refrigerated storage. Second, the washing with massive quantities of a simple 0.9%NaCl/0.2% dextrose solution could also deplete the buffering capacity of the red cell and lower the pH to the point where ATP synthesis is compromised and ATP production no longer exceeds use. Comparison of the initial pH levels, in the current study with its companion where the cells were not frozen, supports this hypothesis. Initial pH in the "liquid" study for the EAS-61 stored cells exceeded 6.9 but was less than 6.6 in the "frozen" study. Though lower for AS-3 stored cells, the differences in initial pH between the "liquid" and "frozen" studies were of the same magnitude. Initial mean pH for AS-3 cells was approximately 6.65 and 6.8 for 100 and 200 mL storage volumes respectively in the "liquid" study but 6.2 and 6.3 in the "frozen" study. Both the initial storage and deglycerolization washing could have contributed to lowering the pH. Perhaps modifying the wash solution with an alkaline buffer would raise the pH sufficiently to boost ATP production. Though ATP concentrations were better maintained in EAS-61, the hemolysis data would favor AS-3 as the superior solution for the final deglycerolization wash and post thaw and deglycerolized storage. It is unknown if a modified wash solution with higher pH would affect hemolysis.

The EAS-61 does not appear to maintain ATP concentrations or minimize hemolysis to the same degree storage of cells in 200 mL of AS-3 under oxygen depleted conditions. See table, which follows for data. However, the establishment and maintenance of an oxygen-depleted environment creates several logistical challenges, which will greatly inhibit adoption on a mass scale or in the clinical or military operations environments.

Table 2: Comparison of Oxygen Depleted and Ambient Stored Red Cells

	Hemolysis:8 Wk (%)	Hemolysis:9 Wk (%)	ATP: 8Wk (% Day 0)	ATP: 9Wk (% Day 0)
AS-3/200 mL: Oxygen Depleted	0.24	0.34	93.9	81.7
EAS-61/200 mL: Ambient	0.5	0.7	59.0	56.5
EAS-61/100 mL: Ambient	1.04	1.36	52.7	44.0
AS-3/200 mL: Ambient	0.85	1.29	52.8	43.1

CONCLUSION:

The superior, red cell preservation qualities of EAS-61 when cells are stored only in the liquid state, is not retained when storing previously frozen cells. The maintenance of an oxygen depleted environment reduces hemolysis and maintains ATP concentrations.

M. "Eleven Week Red Blood Cell Storage in 300 mL of Lower Salt Variants of Bicarbonate Containing EAS-67"

Background:

In cooperation with our collaborator, Dr. Tibor Greenwald from the Hoxworth Blood Center, University of Cincinnati, a series of experiments has been undertaken in which the volume of solution and individual components of the EAS, other than adenine, are being varied individually in order to optimize the quality of the stored red cells. Adenine will not be varied because it took eleven years for the FDA to initially accept the use of 2 mmoL concentrations in red cell storage solutions; raising the adenine issue again would undoubtedly delay FDA licensure unacceptably.

In previous work in Dr. Greenwald's laboratory, two sets of experiments have been completed which led to this protocol. In the first, a series of solutions was tested in which the sodium chloride was incrementally replaced with a millimolar equivalent of sodium bicarbonate. As the sodium chloride was replaced with sodium bicarbonate, the ATP was increased, presumably because a higher pH was maintained longer allowing the glycolytic enzymes to continue producing ATP. Red cell survival averaged > 75% at eleven weeks when 30 mmoL of sodium bicarbonate replaced the equivalent amount of sodium chloride of EAS-64, the solution designated as EAS-67, and 300 mL of the solution was used in storing the cells. EAS-64 has 75 mmoL sodium chloride; the variant EAS-67 had 30 mmoL sodium bicarbonate and only 45 mmoL sodium chloride. Red cell survival was equivalent for the red cells stored in 300 mL of EAS-67 for 11 weeks to red cells stored in 200 mL of EAS-64 for 10 weeks. Hemolysis was not perceptibly different but unacceptably high after 11 weeks of storage.

In another set of experiments, variants of EAS-61 were created in which the sodium chloride concentration was incrementally increased from 26 mmoL to 50, 75 and 150 mmoL. In those solutions, the amount of vesicle protein after nine weeks of storage increased; and therefore, the amount of hemolysis related to hemoglobin trapped in micro vesicles as they are being shed from the surface of the red cell, the process of microvesiculation, likewise increased. However, microvesiculation accounts for only a portion of total hemolysis. In that same experiment, total hemolysis appeared to be lowest and ATP was highest with 50 mmoL of sodium chloride though the differences between 26 and 50 mmoL containing solutions were minimal and not significant. Red cell morphology improved as the sodium chloride concentration decreased. This improvement in morphology is presumed to be the result of hypotonic swelling as predicted by the previous work of Meryman and colleagues.

This set of EAS variants with decreasing sodium chloride content is constructed to determine if the superior survival and ATP production of bicarbonate containing solutions can be retained while lowering the hemolysis. The research questions addressed are:

1. Will ATP content at conclusion of storage in the bicarbonate containing EAS be maintained even as sodium chloride concentrations are reduced?
2. Can hemolysis in the bicarbonate containing EAS be minimized?

The hypothesis was that total hemolysis would decrease and red cell morphological index would increase with decreasing sodium chloride concentration.

METHODS:

Volunteers were recruited under the provisions of WRAIR protocol #776, "The Repeated Use of Small Volumes of Purchased Human Blood in Laboratory Tests, Instrument Calibration and In Vitro Experiments." Twenty-four units were collected; each volunteer donated one unit. Each unit was collected into CP2D and leukocytes removed with the Pall-MEDSEP LEUKOTRAP® whole blood leukoreduction filter. All units were packed to a target 75% hematocrit and pooled in four units' pools of identical ABO group. After mixing, the blood was divided into four aliquots. To one aliquot from each pool, 300 mL of each of four solutions, EAS-67, EAS-W1, EAS-W2 and EAS-W3, was added . The composition of each of the solutions is detailed in the following table. All aliquots were stored at 1 – 6°C and sampled on the

day of preparation and weekly thereafter for 11 weeks. Samples were analyzed for CBC, blood gas analysis (BGA), centrifuged hematocrit, red cell morphology, supernatant hemoglobin, sodium, potassium, and phosphate, in addition to whole blood ATP, glucose and lactate. Percent hemolysis was calculated for the supernatant and total hemoglobin content. The calculated bicarbonate for the BGA was be recorded and included in the data analysis.

Graphical and statistical comparisons of ATP concentrations, red cell morphology and hemolysis of RBC stored in each of the four variants were the primary outcome analyzed. Secondarily, a statistical comparison of additional hematologic and biochemical parameters of RBC stored each of the solution variants was also performed Analysis of variance was performed using the statistical functions available in MS Excel®. Differences with a probability of $p \leq 0.05$ were considered significant.

Table 3: Composition of Bicarbonate-Containing EAS Variants (in meq):

	NaCl	Adenine	Glucose	Mannitol	Na_2HPO_4	$NaHCO_3$	Osmolarity
EAS-67	45	2	50	20	9	30	249
EAS-W1	35	2	50	20	9	30	229
EAS-W2	25	2	50	20	9	30	209
EAS-W3	15	2	50	20	9	30	189

RESULTS:

Examined graphically and measured over eleven weeks of storage, the results from the parameters of greatest interest, i.e., ATP concentration, hemolysis, lactate concentration, pH, morphology, and phosphate concentration, were highly similar with no remarkable differences. The only difference noted among the storage solutions was an increase in calculated mean cell

volume as the NaCl content decreased. Summary data tables and graphs are attached as Appendix 7.

DISCUSSION:

Reducing the NaCl had no apparent effect on the ATP concentrations at the end of storage when the bicarbonate content of the storage solution is constant at 30 meq. Of interest is the pattern of ATP concentrations over time. When adenine containing storage solutions are used, ordinarily the ATP concentrations rise to a peak at 2 - 3 weeks of storage and then fall gradually thereafter. The degree to which the ATP rises and the storage period elapsed before maximal ATP content is achieved varies from one solution to another but the pattern is generally consistent. In this set of experiments, the ATP levels dropped at 7 days of storage and rebounded, reaching maximal levels at 21 - 28 days of storage. When analyzed as percent remaining at end of storage compared to beginning of storage, bicarbonate containing experimental additive solutions appear to sustain relatively higher levels. When the non-bicarbonate containing EAS-61 was used in either 100 or 200 mL of storage solution, the percent remaining averaged 56.5% and 44% respectively after 9 weeks of storage as reported in the protocol described above. By comparison, the bicarbonate containing EAS-67 and variants averaged 65.5 to 70 % of initial levels.

Though ATP appears to have been better maintained, hemolysis was comparable to those obtained with the non-bicarbonate EAS. Hemolysis ranged from 0.46 to 0.52% at 8 weeks and 0.60 to 0.66% at 9 weeks in bicarbonate containing experimental additive solutions. This compares to 0.5 and 0.7% at 8 and 9 weeks respectively for the EAS-61. Hemolysis was unaffected by changes in the NaCl content bicarbonate containing solutions.

CONCLUSION:

ATP concentrations, hemolysis and red cell morphology were unchanged with decreasing sodium chloride content in the bicarbonate containing experimental additive solutions through eleven weeks of storage.

N. "Twelve Week Red Blood Cell Storage in 300 mL of Lower Salt Variants of Bicarbonate Containing EAS-76 after Collection in Alkaline CPD"

STATUS:

This protocol is ongoing as of the end of the fiscal year.

BACKGROUND:

Recently concluded *in vitro* protocols using various formulations of EAS have indicated the potential importance of pH in enhancing ATP synthesis. Also of interest were some of the results from the "frozen" study when compared with the results from the companion "liquid" study. First, the ordinary increase in ATP concentrations observed after 1 - 2 weeks of storage, when non-frozen cells are stored in adenine containing storage solutions, was not seen in the post thaw, deglycerolized cells stored in either AS-3 or EAS-61. ATP concentrations in the "frozen" study were at there highest at the beginning of storage and declined steadily thereafter. Whereas in the liquid study, ATP concentration rose from 0.1 to 0.95 μmol/g Hgb after the beginning of storage, depending upon the storage solution and volume. The second pertinent observation regarded pH at beginning of storage.

The absence of the ATP boost during post thaw storage was unexpected. The most likely explanation is a lowering of the pH during the deglycerolization wash process. All but the final wash step is accomplished with massive quantities of a simple 0.9%NaCl/0.2% dextrose solution. Presumably the buffering capacity of the red cell is depleted to the point where ATP synthesis is compromised to the extent where ATP production no longer exceeds use. Comparison of the initial pH levels, in the current study with its companion where the cells were not frozen, supports that hypothesis. Initial pH in the "liquid" study for the EAS-61 stored cells exceeded 6.9 but was less than 6.6 in the "frozen" study. Though lower for AS-3 stored cells, the differences in initial pH between the "liquid" and "frozen" studies were of the same magnitude. Initial mean pH for AS-3 cells was approximately 6.65 and 6.8 for 100 and 200 mL storage volumes respectively in the "liquid" study but 6.2 and 6.3 in the "frozen" study. Perhaps modifying the wash solution with an alkaline buffer would raise the pH sufficiently to boost ATP production.

Washing the cells with a modified alkaline wash solution was a performed on a single unit using the Haemonetics MDL 21 and AS-3 as the final wash solution and storage solution for the deglycerolized red cells. The standard 0.9% NaCl/0.2% dextrose solution was modified by the addition of 1 meq of disodium phosphate. The initial pH of the cells washed with the modified solution was 6.9 compared to 6.5- 6.6 for the cells washed in the standard solution without the disodium phosphate.

This protocol was designed to collect data in order to answer two research questions. They are:

1. Will the ATP content at conclusion of storage in the bicarbonate containing EAS be increased if the blood is initially collected into an alkalinized CPD?
2. When cells are stored in bicarbonate containing EAS, will the hemolysis differ from that observed when cells are stored in non-carbonate additive solutions?

This protocol is based on the hypothesis that the low pH of the anticoagulant/preservative solution to which the freshly collected cells are exposed suppresses the initial pH and, thereby, compromises ATP production and perhaps also increases hemolysis. Therefore, modifying the pH of the solution could raise the initial pH of the red cells after addition of additive solution.

METHODS:

Volunteers were recruited under the provisions of WRAIR protocol #776, "The Repeated Use of Small Volumes of Purchased Human Blood in Laboratory Tests, Instrument Calibration and In Vitro Experiments." A minimum of twelve units of blood were collected; each volunteer donated one unit. Six were collected into standard, unmodified CPD and six into an alkalinized CPD. The alkalinized CPD was contained in a customized collections system. The techniques of pooling and splitting the pool into aliquots for each treatment group, used with recent *in vitro* protocols, was not used because it is the initial blood collection being varied, not the storage conditions. The composition of both the unmodified and alkalinized CPD is described in a table, which follows. The whole blood was white cell depleted using the Fenwal Sepacell® whole blood leukoreduction filter and packed to a target hematocrit of 75%. Three hundred mL EAS-76 was added to all cells and stored at 1 – 6°C. The composition of EAS-76 is described below. Each of the aliquots was sampled on the day of preparation and weekly thereafter for 12 weeks. Samples

were analyzed for CBC, blood gas analysis (BGA), centrifuged hematocrit, red cell morphology, supernatant hemoglobin, sodium, potassium, and phosphate, in addition to whole blood ATP, glucose and lactate. Percent hemolysis was calculated for the supernatant and total hemoglobin content. The calculated bicarbonate for the BGA was recorded and included in the data analysis.

Table 4: Composition of Unmodified and Alkalinized CPD

	CPD (g/L) pH 5.5 (mM)	**Alkaline CPD** (g/L) pH 8.6 (mM)
Trisodium Citrate•$2H_20$	26.349 / 110	29.333 / 123
Citric Acid•H_20	3.27 / 123	0 / 0
Dextrose•H_20	25.556 / 130	25.556 / 130
Monosodium Phosphate•H_20	2.222 / 16	0 / 0
Disodium Phosphate•$7H_20$	0 / 0	4.25 / 16

Table 5: Composition of EAS-76:

Chemical	mM	g/L
NaCl	45	2.63
Adenine	2	0.27
Dextrose	50	9.008
Mannitol	30	5.465
Na_2HPO_4	9	2.413
$NaHCO_3$	30	2.52

The primary outcomes to be analyzes are the difference in ATP concentrations and hemolysis of RBC collected into the standard CPD versus an alkalinized CPD. Secondarily, comparison of additional hematologic and biochemical parameters of RBC collected into the standard CPD versus an alkalinized CPD will be made using analysis of variance. Statistical probabilities of $p \leq 0.05$ will be considered significant.

RESULTS, DISCUSSION and CONCLUSION:

This protocol was not completed and no interim data analysis was made as of end of the fiscal year. Therefore, no results were available.

GENERAL AND ADMINISTRATIVE:

There have been no significant administrative changes in contract operation since the previous annual report. Instead, existing systems and processes for documentation, equipment maintenance and repair, and laboratory testing procedure validation have been maintained to meet research requirements.

Manuscripts of research results from contract supported research have been submitted for publication with some published during the report period. The status of each manuscript submission is detailed in previous sections of this report.

In June, the Institute Director announced the intended relocation of WRAIR-based, combat casualty care research activities, including Blood Research, to the Institute of Surgical Research in San Antonio Texas. There were some indications would not accompany the other WRAIR activities but would be delayed. The timing of neither relocation were specified and are unknown.

SUMMARY

The contract staff has supported the BRD by operating and maintaining the Blood Storage Laboratory to support both existing and new requirements. The staff is trained and systems are in place which support red cell survival protocols. The Laboratory has been successful in completing protocols and having its work accepted for publication in peer-reviewed scientific journals during fiscal year 2000. The BRD mission has been supported.

REFERENCES

1. Chapman KW, Snell SM, Jesse RG, Morano JK, Everse J, and Winslow RM. Pilot Scale Production of Pyrogen-Free Human Hemoglobin for Research, Biomat., Art. Cells & Immob. Biotech. 1992: 20(2-4):415-421.

2. Nelson D, et.al. Preparation and Characterization of Diaspirin Cross-Linked Hemoglobin Solutions for Preclinical Studies, Biomat., Art. Cells & Immob. Biotech. 1992: 20(2-4):423-427.

3. Winslow RM and Chapman KW. Pilot-Scale Preparation of Hemoglobin Solutions, Methods in Enzymology. 1994: 231:3-16.

4. Farmer M, Ebeling A, Marshall T, Hauck W, Sun CS, White E, Long Z. Validation of Virus Inactivation by Heat Treatment in the Manufacture of Diaspirin Cross linked Hemoglobin, Biomat., Art. Cells & Immob. Biotech. 1992: 20(2-4):429-433.

5. Cliff RO, Ligler F, Goins B, Hoffmann PM, Speilberg H, Rudolph AS. Liposome Encapsulated Hemoglobin: Long-Term Storage Stability and In Vivo Characterization, Biomat., Art. Cells & Immob. Biotech. 1992: 20(2-4):619-626.

6. Rudolph AS. Encapsulation of Hemoglobin in Liposomes, Blood Substitutes: Physiological Basis of Efficacy, 1995; 7:90-104.

7. Meryman HT, Hornblower M, Syring RL. Prolonged Storage of Red Cells at 4°C. Transfusion. 1986; 26:500-505.

8. Meryman HT, Hornblower M, Syring RL, Mesbah-Karimi N. Extending the Storage of Red Cells at 4°C. Transfusion Science. 1994; 15:105-115.

9. Greenwalt TJ, McGuinness CG, Dumaswala UJ, Carter HW. Studies in Red Blood Cell Preservation 3. A Phosphate-Ammonium-Adenine Additive Solution. Vox Sanguinis. 1990; 58:94-99.

10. Dumaswala UJ, Oreskovic RT, Petrosky TL, Greeenwalt TJ. Studies in Red Cell Preservation 5. Determining the Limiting Concentrations of NH_4Cl and Na_2PO_a Needed to Maintain Red Blood Cell ATP During Storage. Vox Sanguinis. 1992; 62:136-140.

11. Hess JR., Rugg N, Knapp AD, Gormas JF, Silberstein EB, Greenwalt TJ. Successful storage of RBCs for 9 weeks in a new additive solution. Transfusion 2000; 40:1007-1011.

12. Hess JR., Rugg N, Knapp AD, Gormas JF, Silberstein EB, Greenwalt TJ. Successful storage of RBCs for 10 weeks in a new additive solution. Transfusion 2000;40:1012-1016.

13. Surgeoner D, Klein H, Institute of Medicine Conference on Blood Safety and Availability, 9 June 1995, Washington, DC.

14. Donor Selection and Blood Collection. In Vengelen-Tyler V, Ed. Technical Manual, 12th Edition, American Association of Blood Banks, Bethesda, MD, 1996:73-102.

15. Code of Federal Regulations, Title 21 Section 640, U.S. Government Printing Office

16. Moroff G, Sohmer PR, Button LN, et. al. Proposed standardization of methods for determining the 24-hour survival of stored red cells. Transfusion 1984;24:109-114.

17. Babcock, JG, Lippert LE, Derse-Anthony CP, Mechling M, Hess JR., A hypotonic storage solution did not prolong the viability of RBCs. Transfusion 2000:40:994-999.

18. AuBuchon JP, Estep TN, Davey RJ. The effect of the plasticizer di-2-ethylhexyl phthalate on the survival of stored RBC's. Blood 1988;71(2):448-452.

19. Labow RS, Card RT, Rock G. The effect of the plasticizer di-2-ethylhexyl phthalate on red cell deformability. Blood 1987;70(1):319-323.

20. Estep TN, Pedersen RA, Miller TJ, Stupar KR. Characteristics of erythrocyte quality during refrigerated storage of whole blood containing di-(2-ethylhexyl) phthalate. Blood 1984:64(6)1270-1276.

21. Myhre BA, Johnson D, Marcus CS, et.al. Survival of red cells stored for 21 and 35 days in a non-di-(2-ethylhexyl) phthalate plastic container. Vox Sang 1987; 53:199-202.

22. Rock G, Tocchi M, Ganz PR, Tackberry ES. Incorporation of plasticizer into red cells during storage. Transfusion 1984;24(6)493-498.

23. Yoshita T, Biensky MW, Pickerd CA, Herschel LH, Roger J, AuBuchon JP. 9-week storage of red blood cells in AS-3 under oxygen depleted conditions. Transfusion 1999;39S:109S.

Appendix 1

Contract Employees as of end of Fiscal Year 1999:

Lloyd E. Lippert, Ph.D., SBB(ASCP), Project Manager (Full-time)

Cynthia Oliver, B.S., MT(ASCP) SBB, Technologist (Full-time)

Heather Hill, B.S.,M.S., Technologist (Full-Time)

Patricia Cowan, BSN, RN, Registered Nurse (Part-time)

Nicole Putnam-Frenchik, Administrative Assistant (Part-time)

Appendix 2

Title: A Multicenter Study of In Vitro and In Vivo Parameters of Human Red Blood Cells Frozen with 40 Percent W/V Glycerol and Stored After Deglycerolization for 15 Days at 4 C in AS-3: Assessment of RBC Processing in the Haemonetics Model 215.

Authors: Valeri CR, Ragno G, Pivacek LE, Srey R, Hess JR, Lippert LE, Mettille F, Fahie R, O'Neill EM, and Szymanski IO.

Haemonetics 215 MS 9/22/00

A MULTICENTER STUDY OF IN VITRO AND IN VIVO PARAMETERS OF HUMAN RED BLOOD CELLS FROZEN WITH 40 PERCENT W/V GLCEROL AND STORED AFTER DEGLYCEROLIZATION FOR 15 DAYS AT 4 C IN AS-3: ASSESSMENT OF RBC PROCESING IN THE HAEMONETICS MODEL 215

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Running Title: Automated, functionally closed system to glycerolize, deglycerolize, and store red blood cells at 4 C in AS-3 for 15 days

ABSTRACT

Background: The FDA has approved the storage of frozen red blood cells (RBC) at -80 C for 10 years. Following deglycerolization the RBC can be stored at 4 C for no more than 24 hours because open systems are currently being used in the glycerolizing and washing procedures, introducing the potential for bacteriologic contamination. Our laboratory has been evaluating the Haemonetics Model 215, an automated functionally closed system, used for both glycerolization and deglycerolization processes. We report here on a multicenter study to assess the quality and sterility of RBC processed using this instrument.

Study Design and Methods: Studies were performed at three military sites and two civilian sites, each performing in vitro testing of approximately 20 units of RBC. Additionally, at one military site and at two civilian sites, in vivo studies were conducted. At each site, about

ten units of RBC that had been stored at 4 C in AS-3 for 15 days after deglycerolization were autotransfused.

At one of the civilian sites, NBRL, each of ten volunteers was autotransfused on two occasions in a randomized manner, once with previously frozen RBC that had been stored at 4 C in AS-3 for 15 days after deglycerolization and once with liquid-preserved RBC that had been stored at 4 C in AS-1 for 42 days.

Measurements were made of in vitro RBC recovery, hemolysis and bacteriologic cultures. Twenty-four-hour posttransfusion survival values were assessed by double label procedures and the 51Cr T-1/2 values were measured.

Results: For 142 units studied at the five different sites, the mean in vitro freeze-thaw-wash (FTW) recovery value was 87 percent ± 5 (SD); the mean supernatant osmolality on the day of deglycerolization was 297 mOsm/kg H_2O ± 5 (SD), and

the mean percent hemolysis after storage at 4 C in AS-3 for 15 days was 0.60 percent ± 0.2 (SD). At three sites at which 24-hour posttransfusion survival values were measured by three different double label procedures, a mean 24-hour posttransfusion survival of 77 percent ± 9 (SD) was observed for a total of 36 autotransfusions in 12 females and 24 males of previously frozen RBC that had been stored at 4 C in AS-3 for 15 days following deglycerolization.

Conclusions: The multicenter study demonstrated the acceptable quality of RBC glycerolized and deglycerolized in the automated Haemonetics Model 215 instrument.

Key Words: Functionally closed automated system to glycerolize and deglycerolize human red cells; in vitro recovery; hemolysis; sterility; 24-hour posttransfusion survival values using double label procedures and 51Cr T-1/2 values

INTRODUCTION

Previously frozen RBC are FDA-approved for storage at -80 C for 10 years. Following deglycerolization the RBC can be stored at 4 C for no more than 24 hours because the current methods for glycerolization and deglycerolization are open systems with the potential for bacteriologic contamination. The Haemonetics Model 215 instrument (Haemonetics Corp., Braintree, MA) is an automated, functionally closed system for the glycerolization and deglycerolization of human RBC, utilizing a sterile connector device (SCD), in-line 0.22 micron filters to deliver solutions, a disposable polycarbonate bowl with an external seal and an integrally attached shaker and a printer to record the glycerolization and deglycerolization processes. The quality of the RBC is monitored by an optical system that measures the hemoglobin concentration in the waste solution during the deglycerolization procedure.

This multicenter study was done to assess the quality of RBC glycerolized and deglycerolized in the automated Haemonetics Model 215.

Studies at the five sites were conducted over a 2-year period. The three military locations were Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD, Naval Medical Center Portsmouth (NMC Portsmouth), Portsmouth, VA, Naval Medical Center Great Lakes (NMC Great Lakes), Great Lakes, IL. The two civilian sites were the Naval Blood Research Laboratory (NBRL), Boston University School of Medicine, Boston, MA, and the University of Massachusetts Memorial Health Care (UMASS), Worcester, MA. At each of the five sites, in vitro studies were done on 17 to 28 units of RBC. About ten autotransfusions were performed at each of the two civilian locations (NBRL and UMASS) and at one military location (WRAIR), and 24-hour posttransfusion survivals were calculated using double isotope procedures.

The NBRL also conducted a randomized study in which each of ten volunteers was autotransfused on two separate occasions, once with previously frozen deglycerolized RBC that had been stored at 4 C in AS-3 for 15 days and another time with liquid-preserved RBC that had been stored at 4 C in AS-1 for 42 days.

MATERIALS AND METHODS

One hundred and sixty-five (165) healthy male and female volunteers who met the requirements of the American Association of Blood Banks for acceptable blood donors each donated a unit of whole blood. In addition to in vitro studies, 12 female volunteers who were post-menopausal or had been surgically sterilized and 24 male volunteers participated in autotransfusion studies.

The study was reviewed and approved by the Institutional Review Board for Human Research, Boston University Medical Center, Boston, MA, the Human Use Review Committee, WRAIR, Washington, DC, Clinical Investigation and Research Department, Naval Medical Center Portsmouth, Portsmouth, VA, Clinical Investigation and Research Department, Naval Medical Center Great Lakes, Great Lakes, IL, and the Committee for the Protection of Human Subjects in Research, University of Massachusetts Medical Center,

Worcester, MA. Informed consent was obtained from each volunteer.

From volunteers who had a hematocrit value of at least 40 V percent and not higher than 46 V percent, 450 grams of whole blood were collected. From volunteers whose hematocrit values were greater than 46 V percent, 430 grams of blood were collected. The blood was collected into 63 ml of CPDA1 anticoagulant in the 800 ml PVC plastic bag with a ratio of eight (8) volumes of blood to one (1) volume of CPDA1 anticoagulant. A volume of RBC not greater than 180 ml was collected since this is the maximum volume that can be deglycerolized in the 275 ml volume disposable bowl used in the Haemonetics Model 215 instrument.

The CPDA1 whole blood was stored for up to 24 hours at 4 C, after which it was centrifuged at 1615 X g for 4 minutes and the plasma removed to produce a hematocrit of 75 ± 5 (SD) V percent. The RBC were stored at 4 C for a total

of 6 days prior to glycerolization and freezing. Using a disposable glycerolization harness with an in-line 0.22 micron filter in the Haemonetics Model 215, the RBC were glycerolized with a volume of 6.2 M glycerol solution to achieve a final glycerol concentration of 40 percent W/V. The glycerolized RBC were centrifuged at 1250 X g for 10 minutes and the supernatant solution removed (1). The 800 ml PVC plastic bag containing the glycerolized RBC concentrate was overwrapped in a polyester plastic bag that was sealed and placed in a cardboard box. The glycerolized RBC were frozen and stored in a -80 C mechanical freezer for at least 4 weeks.

During thawing in the 42 C water bath (Blue-M) with a circulating pump, the frozen RBC were kept in the plastic overwrap to avoid bacterial contamination. To reduce the thawing time when the 36 C plasma thawer was used (Thermogenesis, Rancho Cordoba, CA), the plastic bag

overwrap was not used because these RBC were not directly exposed to water. After thawing to a surface temperature of 34 C, monitored by an infrared scanner (Exergen Corp., Watertown, MA), the RBC were deglycerolized using the SCD and the disposable set of the Haemonetics Model 215. Each unit was deglycerolized in the instrument's 275 ml blow-molded disposable bowl with an external seal using 50 ml of 12 percent NaCl, 1.6 liters of 0.9 percent NaCl-0.2 percent glucose, and 250 ml of additive solution-formula 3 (AS-3 solution) (Haemonetics Corp., Braintree, MA).

The Haemonetics Model 215 has a printer attached to the instrument which records the glycerolization and deglycerolization procedures. In addition, a graph records the hemolysis measured during the deglycerolization process. Total hemoglobin concentration was measured by the cyanmethemoglobin method; the hematocrit was measured using the microhematocrit method; and supernatant hemoglobin was

measured using a spectrophotometric method (Spectronic Instruments, Inc., Syracuse, NY) (2). Osmolality was measured using a wide range osmometer (Fiske Model 2400, Advanced Instruments, Norwood, MA), and intracellular and extracellular sodium and potassium ions were measured using a flame photometer (Model 943, Instrumentation Laboratory, Lexington, MA) (3). The percent hemolysis was calculated by dividing the total supernatant hemoglobin by the combination of the total cellular hemoglobin and total supernatant hemoglobin. Using aerobic blood agar and thioglycollate broth and anaerobic tryptic soy broth, bacterial cultures were performed on the previously frozen RBC stored in AS-3 at 4 C for 15 days on the day of deglycerolization, three days prior to the autotransfusion and on the day of the autotransfusion. Cultures were done at both NBRL, Boston, MA and the Microbiology Laboratory at UMASS, Worcester, MA. Previously frozen RBC stored at 4 C in AS-3 solution for 42

days and then at 22 C for 30 days were cultured after each storage period. Cultures were also performed on the liquid-preserved RBC stored for 42 days at 4 C in AS-1 three days before and on the day of autotransfusion. The blood agar plate and thioglycollate broth tubes were incubated at 37 C and examined for growth for 7 days.

Freeze-thaw-wash (FTW) recovery values were calculated by comparing the total hemoglobin in the RBC after thawing to the total cellular hemoglobin after washing. WBC counts were done using the Coulter Counter STKS and the Nageotte hemocytometer and Turk's solution, both on the day of deglycerolization and after storage at 4 C in AS-3 solution for 15 days (4,5).

At each of the five sites (NBRL, WRAIR, NMC Portsmouth, NMC Great Lakes, UMASS), in vitro testing was done on about 20 units of previously frozen RBC after postwash storage at 4 C in AS-3 solution for 15 days. Measurements were made of

freeze-thaw (FT) and freeze-thaw-wash (FTW) recovery values, hemoglobin concentration, RBC count, platelet count, hematocrit value, supernatant hemoglobin level, supernatant potassium level, and supernatant osmolality.

At NBRL 14 volunteers were autotransfused previously frozen RBC that had been stored at 4 C in AS-3 solution for 15 days after deglycerolization. Ten of these 14 volunteers participated in a randomized study in which they also were autotransfused liquid-preserved RBC stored at 4 C in AS-1 for 42 days. In this randomized study, the 51Cr/125I albumin double label procedure was used to measure the 24-hour posttransfusion survival (6-8). The volunteer's RBC volume was measured indirectly from the I-125 albumin plasma volume and total body hematocrit estimated from the peripheral venous hematocrit multiplied by 0.89. Blood samples were collected 5, 10, 15, 20 and 30 minutes and 24 hours following the autotransfusion to measure the

posttransfusion survival values of the 51Cr-labeled previously frozen and liquid-preserved RBC. The 100 percent survival value was calculated from the 51Cr radioactivity associated with the autotransfused RBC and the recipient's RBC volume. The survival of the 51Cr autologous preserved RBC was a percentage of the 100 percent survival value (6).

In four females and four males studied at WRAIR who were autotransfused 51Cr-labeled RBC that had been stored at 4 C in AS-3 solution for 15 days, 24-hour posttransfusion survival was measured by the 51Cr/99mTc double label procedure in which the previously frozen RBC were labeled with 51Cr and the recipient's RBC volume was measured with 99mTc fresh autologous RBC (8,9).

In six male and eight female volunteers autotransfused at UMASS, the 24-hour posttransfusion survival of previously frozen RBC that had been stored at 4 C for 15 days following deglycerolization was measured using the double 51Cr

procedure. Two (2) microcuries of 51Cr were used to label fresh autologous RBC for the RBC volume measurement, and 20 microcuries of 51Cr were used to label the previously frozen RBC (10). The index of therapeutic effectiveness was calculated from the freeze-thaw-wash recovery multiplied by the 24-hour posttransfusion survival value (1).

Statistical Analyses. Statistical analyses were performed using SAS® (SAS Institute, Inc., Cary, NC). A paired t test was used to test for significant differences in results of a randomized study in which ten volunteers were autotransfused previously frozen RBC stored at 4 C in AS-3 for 15 days following deglycerolization and liquid-preserved RBC stored in CPD/AS-1 at 4 C for 42 days. A P value of 0.05 was considered significant. Regression was used to describe the posttransfusion survival of the autologous RBC. Means and standard deviations (SD) are reported.

RESULTS

A total of 165 units of blood were collected at five sites and frozen using the Haemonetics 215 instrument. Twenty-three units were excluded from analyses: one unit was contaminated, nine units were frozen less than 2 weeks, two units were stored in a 4 C refrigerator that malfunctioned, three units leaked during thawing from improper sealing of the tubing, one unit had a technical problem with radioactive labeling, in two cases the full unit was not recovered in processing, one unit of deglycerolized RBC was stored at 4 C in AS-3 for 20 days instead of 15 days, three recipients did not return for reinfusion, and one recipient had poor venous access and could not be reinfused.

In vitro results were obtained on 142 of the 165 units collected. In vivo 24-hour posttransfusion survivals and 51Cr lifespan values were measured in 36 subjects who

received previously frozen RBC stored at 4 C in AS-3 for 15 days following deglycerolization and in ten subjects who received autologous liquid-preserved RBC stored at 4 C in AS-1 for 42 days.

In vitro measurements in the 142 units showed freeze-thaw recovery values of 98.6 percent ± 1.3 (SD) and freeze-thaw-wash recovery values of 87.0 percent ± 5 (SD) (Tables 1 and 2). The previously frozen RBC stored in AS-3 at 4 C for 15 days showed a mean percent hemolysis of 0.6 ± 0.2 (Tables 1 and 2).

Aerobic and anaerobic cultures showed no bacteriologic growth in 141 of the 142 units of previously frozen RBC cultured after storage at 4 C in AS-3 solution for 15 days following deglycerolization. One in vitro unit grew the skin contaminant, Proprionibacterium Acnes, on day 15 and on day 42. One of the ten liquid-preserved units that were

stored in CPD/AS-1 at 4 C for 42 days also grew Proprionibacterium Acnes following incubation at 37 C for 3 days (Table 3), but culture of this unit on day 39 showed no growth. These findings suggest that contamination of the previously frozen RBC occurred at the time of collection and contamination of the liquid-preserved RBC occurred when the culture sample was collected on day 42.

In the 14 volunteers in the NBRL study in which the double label procedure was used to measure the 24-hour posttransfusion survival of previously frozen RBC stored in AS-3 for 15 days following deglycerolization, the survival value was 75 ± 7 percent (SD), and in 13 of these volunteers, the 51Cr T-1/2 value was 29 ± 4 days (Table 4). In ten of these 14 in whom liquid-preserved RBC stored in AS-1 for 42 days at 4 C also were autotransfused, the 24-hour posttransfusion survival value was 72 ± 5 percent (SD)

using the double label procedure, and the 51Cr T-1/2 value was 27 ± 3 days (Tables 4 and 5).

At WRAIR the 51Cr/99mTc double label procedure was used for autotransfusions to eight volunteers. The deglycerolized RBC stored at 4 C in AS-3 solution for 15 days were labeled with 51Cr and fresh autologous RBC were labeled with 99mTc to measure the RBC volume. The mean 24-hour posttransfusion survival value was 78 ± 9 percent (SD), and for seven of the eight, the 51Cr T-1/2 value was 24 ± 5 days.

At UMASS, the double label procedure for 14 autotransfusions of previously frozen RBC that had been stored in AS-3 for 15 days following deglycerolization was used. The 24-hour posttransfusion survival value was 78 ± 10 percent (SD) and the 51Cr T-1/2 value was 29 ± 5 days (Table 4). The mean index of therapeutic effectiveness

(ITE) for the 36 autotransfusions performed at three of the study sites was 68 ± 8 (SD) percent for the previously frozen RBC and 72 ± 5 percent (SD) for the ten autotransfusions performed at one site of liquid-preserved RBC stored in CPD/AS-1 at 4 C for 42 days.

The Nageotte chamber and Turk's solution was used to measure WBC counts both on the day of deglycerolization and following storage at 4 C in AS-3 solution for 15 days, and at both times the total number of WBC was 1×10^7 per unit, with a range from 1×10^6 to 4×10^7 for 73 units (Tables 1 and 2).

DISCUSSION

The Haemonetics Model 215 is a functionally closed automated instrument for glycerolization and deglycerolization of human RBC. This instrument uses a disposable blow-molded 275 ml bowl with a diverter, an external rotating seal, and a capacity to process 180 ml of RBC. If the volume exceeds 180 ml, the RBC will spill over into the waste solution during the deglycerolization procedure.

Previously frozen RBC stored at 4 C in AS-3 solution for 15 days following deglycerolization were shown to have a mean 24-hour posttransfusion survival value of 77 ± 9 percent (SD) and 0.6 ± 0.2 percent hemolysis. Ten units of liquid-preserved RBC stored in AS-1 at 4 C for 42 days exhibited a mean 24-hour posttransfusion survival value of 72 percent ± 5 (SD) when measured by the 51Cr/125I albumin

double label procedure, a finding similar to that observed in a NBRL study in 1988 (7), and not significantly lower than the mean 24-hour posttransfusion survival value of 77 percent ± 9 (SD) observed for the 36 autotransfusions in the present study of deglycerolized RBC stored at 4 C in AS-3 for 15 days and measured by the 51Cr/125I albumin, 51Cr/99mTc, and double 51Cr label procedures.

One in vitro unit of deglycerolized RBC grew Proprionibacterium Acnes bacteria after storage at 4 C in AS-3 solution for 15 days. One unit from a separate donor stored in the liquid state in CPD/AS-1 at 4 C for 42 days that tested positive for the Proprionibacterium Acnes bacteria showed no growth in a sample tested three days previously on day 39. These findings suggest that the skin contaminant seen in the deglycerolized RBC occurred at the time of blood collection and that contamination of the liquid-preserved RBC occurred during sample collection on

day 42. Information provided from the ongoing bacterial contamination study of blood by the American Association of Blood Banks (AABB), American Red Cross (ARC), Centers for Disease Control (CDC) and Department of Defense (DOD) (BaCon study) indicates that this bacterium has not been associated with posttransfusion sepsis.

Concern about the transmission of disease from allogeneic RBC has led to the call for quarantine of donor RBC and subsequent retesting of the donor. Frozen RBC can be stored for at least ten years. If the blood donor continues to test negative for infectious disease markers over a six (6) month period, the frozen RBC can be considered safe for allogeneic transfusion. The ability to store the deglycerolized RBC at 4 C for at least two weeks makes this practice even more valuable.

In previous studies the processes of glycerolizing and deglycerolizing RBC in the presence and absence of freezing

was shown to reduce the total number of WBC in the deglycerolized unit (11-17). In the study reported here, measurements made using the Nageotte chamber and Turk's solution in 73 units on the day of deglycerolization and after postwash storage at 4 C in AS-3 for 15 days demonstrated that the freeze-thaw-wash process reduced the total number of WBC to a mean of 1×10^7 per unit, with a range from 1×10^6 to 4×10^7 (4,5). With leukoreduction, the total number of WBC in the unit is presumed to be 5×10^6 or less, although this is an arbitrary level not supported by scientific data. Only human studies can demonstrate for certain whether using filtration for leukoreduction to a total WBC count of 5×10^6 or less will produce a significantly greater reduction in transmission of cytomegalovirus and other infectious agents, immune suppression, and febrile non-hemolytic transfusion reactions

than the freeze-thaw-wash procedure that produces a total WBC count of 1 X 10^7 in deglycerolized RBC (18-30).

Washing previously frozen RBC to remove the cryoprotectant, glycerol, in addition to reducing the number of WBC in the unit, also reduces the number of biologically active substances that some investigators believe may contribute to the immunomodulation effects in recipients (31-35). In addition to the number of WBC (18-30) and the biologically active substances (31-35), the number of compatible nonviable RBC (7) may also be responsible for transfusion-induced immunomodulation.

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REFERENCES

1. Valeri CR, Valeri DA, Anastasi J, Vecchione JJ, Dennis RC, Emerson CP. Freezing in the primary polyvinylchloride plastic collection bag: A new system for preparing and freezing nonrejuvenated and rejuvenated red blood cells. Transfusion 1981;21:138-149.

2. Blakney GB, Dinwoodie AJ. A spectrophotometric scanning technique for the rapid determination of plasma

3. Runck AH, Valeri CR, Sampson WT. Comparison of the effects of ionic and non-ionic solutions on the volume and intracellular potassium of frozen and non-frozen human red cells. Transfusion 1968;8:9-18.

4. Greenwalt TJ, Allen CM. A method for counting leukocytes in filtered components. Transfusion 1990; 30:377-379.

5. Lutz P, Dzik WH. Large-volume hemocytometer chamber for accurate counting of white cells (WBCs) in WBC-reduced platelets: validation and application for quality control of WBC-reduced platelets prepared by apheresis and filtration. Transfusion 1993;33:409-412.

6. Valeri CR, Pivacek LE, Ouellet R, Gray A. A comparison of methods of determining the 100 percent survival of preserved red cells. Transfusion 1984;24:105-108.

7. Valeri CR, Pivacek LE, Palter M, Dennis RC, Yeston N, Emerson CP, Altschule MD. A clinical experience with ADSOL-preserved erythrocytes. Surg Gynec Obstet 1988; 166:33-46.

8. Davey RJ. Red cell radiolabeling and kinetics. In: Rossi EC, Simon TL, Moss GS, Gould SA, eds. Principles of Transfusion Medicine, 2nd edition, Baltimore: Williams and Wilkins, 1996:43-50.

9. Reid TJ, Babcock JG, Derse-Anthony CP, Hill HR, Lippert LE, Hess JR. The viability of autologous human red cells stored in additive solution 5 and exposed to 25 C for 24 hours. Transfusion 1999;39:991-997.

10. Szymanski IO, Valeri CR. Evaluation of double 51Cr technique. Vox Sang 1968;15:287-292.

11. Valeri CR. Blood Banking and the Use of Frozen Blood Products, Chemical Rubber Company Press, Boca Raton, FL, 1976.

12. Crowley JP, Valeri CR. The purification of red cells for transfusion by freeze-preservation and washing. II. The residual leukocytes, platelets, and plasma in washed, freeze-preserved red cells. Transfusion 1974; 14:196-202.

13. Crowley JP, Valeri CR. The purification of red cells for transfusion by freeze-preservation and washing. III. Leukocyte removal and red cell recovery after red cell freeze-preservation by the high or low glycerol concentration method. Transfusion 1974;14:590-594.

14. Crowley JP, Skrabut EM, Valeri CR. Immunocompetent lymphocytes in previously frozen washed red cells. Vox Sang 1974;26:513-517.

15. Crowley JP, O'Donnell M, Sell KW, Valeri CR. The purification of red cells for transfusion by freeze-preservation and washing. IV. The use of micropore filtration to reduce the residual HL-A antigenicity of previously frozen, washed red cells. Transfusion 1975; 15:34-38.

16. Kurtz SR, Van Deinse WH, Valeri CR. The immunocompetence of residual lymphocytes at various stages of red cell cryopreservation with 40% W/V glycerol in an ionic medium at -80 C. Transfusion 1978;18:441-447.

17. Kurtz SR, Valeri DA, Melaragno AJ, Gray A, Vecchione JJ, Emerson CP, Valeri CR. Leukocyte-poor red blood cells prepared by the addition and removal of glycerol from red blood cell concentrates stored at 4 C. Transfusion 1981;21:435-442.

18. British Committee on Standards in Haematology. Guidelines on the clinical use of leucocyte-depleted blood components. Transfu Med 1998;8:59-71.

19. Danish Society of Clinical Immunology. Danish recommendations for the transfusion of leukocyte-depleted blood components. Vox Sang 1996;70:185-186.

20. Menitove JE, ed. Standards for blood banks and transfusion services, 19th ed. Bethesda, MD: American Association of Blood Banks, 1999;27:53.

21. Food and Drug Administration. Memorandum: Recommendations for licensure requirements for leukocyte-reduced blood products. May 29, 1996. Rockville, MD: CBER Office of Communications, Training, and Manufacturers Assistance, 1996.

22. Vamvakas EC, Blajchman MA. A proposal for an individual patient data based meta-analysis of randomized controlled trials of allogeneic transfusion and postoperative bacterial infection. Transfusion Med Rev 1997;11:180-194.

23. Vamvakas EC. Meta-analysis of randomized controlled trials of the efficacy of white cell reduction in preventing HLA-alloimmunization and refractoriness to random-donor platelet transfusions. Transfusion Med Rev 1998;12:258-270.

24. Dzik S, AuBuchon J, Jeffries L, Kleinman S, Manno C, Murphy MF, Popovsky MA, Sayers M, Silberstein LE, Slichter SJ, Vamvakas EC. Leukocyte reduction of blood components: Public policy and new technology. Transfusion Med Rev 2000;14:34-52.

25. van de Watering LMG, Hermans J, Houbiers JGA, van den Broek PJ, Bouter H, Boer F, Harvey MS, Huysmans HA, Brand A. Beneficial effects of leukocyte depletion of transfused blood on post-operative complications in patients undergoing cardiac surgery. A randomized clinical trial. Circulation 1998;97:562-568.

26. Bordin JO, Chiba AK, Carvalho KIL, Takata ET, Falcao RP, Garcia AB, Bordin IAS, Maciel MM, Andreoni S, Kerbauy J. The effect of unmodified or prestorage white cell-reduced allogeneic red cell transfusions on the immune responsiveness in orthopedic surgery patients. Transfusion 1999;39:718-723.

27. Kao KJ. Mechanisms and new approaches for the allogeneic blood transfusion-induced immunomodulatory effects. Transfusion Med Rev 2000;14:12-22.

28. Vamvakas EC, Blajchman MA. Prestorage versus poststorage white cell reduction for the prevention of the deleterious immunomodulatory effects of allogeneic blood transfusion. Transfusion Med Rev 2000;14:23-33.

29. Blajchman MA. Transfusion-associated immunomodulation and universal white cell reduction: are we putting the cart before the horse? (editorial). Transfusion 1999;39:665-670.

30. Holland PV. Putting the cart before the horse (Letter to the Editor). Transfusion 2000;40:254-256.

31. Nielsen HJ. Detrimental effects of perioperative blood transfusion. Br H Surg 1955;82:582-587.

32. Nielsen HJ, Edvardsen L, Vangsgaard K, Dybkjar E, Skov PS. Time-dependent histamine release from stored human blood products. Br J Surg 1996;83: 259-262.

33. Nielsen HJ, Reimert C, Pedersen AN, et al. Time-dependent, spontaneous release of white cell- and platelet-derived bioactive substances from stored human blood. Transfusion 1996;36:960-965.

34. Shanwell A, Kristiansson M, Remberger M, Ringden O. Generation of cytokines in red cell concentrates during storage is prevented by prestorage white cell reduction. Transfusion 1997;37:678-684.

35. Hyllner M, Arnestad JP, Bengtson JP, Rydberg L, Bengtsson A. Complement activation during storage of whole blood, red cells, plasma, and buffy coat. Transfusion 1997;37:264-268.

TABLE 1

RED BLOOD CELLS PROCESSED IN THE HAEMONETICS MODEL 215 FOR IN VITRO ANALYSES
COMBINED DATA FROM FIVE TEST SITES

			POSTWASH STORAGE AT 4 C		
		POSTTHAW	DAY 0	DAY 1	DAY 15
FREEZE-THAW RECOVERY (%)	MEAN	98.7	---	---	---
	SD	1.1	---	---	---
	N	106	---	---	---
FREEZE-THAW-WASH RECOVERY (%)	MEAN	---	86.8	---	---
	SD	---	5	---	---
	N	---	106	---	---
HEMATOCRIT (V%)	MEAN	61	53	53	51
	SD	5	3	3	3
	N	106	106	106	106
HEMOGLOBIN (gm/dl)	MEAN	17.6	14.4	14.5	14.4
	SD	1.6	1.1	1.1	1.2
	N	106	106	106	106
RBC COUNT ($X10^9$/ml)	MEAN	5.9	4.8	4.8	4.8
	SD	0.6	0.4	0.4	0.4
	N	105	106	106	106
WBC COUNT ($X10^6$/ml)	MEAN	1.8	<0.5	---	---
	SD	0.7	0	---	---
	N	99	105	---	---
MANUAL WBC COUNT (#/ul)	MEAN	---	---	32.5	31.4
	SD	---	---	26.4	25.1
	N	---	---	64	64
TOTAL WBC ($X10^6$ /UNIT)	MEAN	540	---	9.7	9.4
	SD	210	---	7.9	7.5
	N	99	---	64	64
PLATELET COUNT ($X10^6$/ml)	MEAN	54	2	1	1
	SD	43	7	1	1
	N	105	106	105	89
BLOOD pH AT 22 C	MEAN	6.9	6.4	6.4	6.3
	SD	0.1	0.1	0.1	0.1
	N	75	96	100	106
SUPERNATANT OSMOLALITY (mOsm/kg H_2O)	MEAN	4579	296	297	300
	SD	295	4	5	4
	N	106	106	106	106
SUPERNATANT K+ (mEq/l)	MEAN	14.3	1.3	6.9	24.5
	SD	4.0	0.9	3.0	4.9
	N	94	106	106	106
SUPERNATANT HB (mg/dl)	MEAN	607	40	91	174
	SD	495	13	31	51
	N	105	106	106	106
% HEMOLYSIS	MEAN	---	0.1	0.3	0.6
	SD	---	0	0.1	0.2
	N	---	106	106	106

TABLE 2

RED BLOOD CELLS PROCESSED IN THE HAEMONETICS MODEL 215 FOR IN VIVO ANALYSES
COMBINED DATA FROM THREE TEST SITES

		POSTTHAW	POSTWASH STORAGE AT 4 C DAY 0	DAY 15
FREEZE-THAW RECOVERY (%)	MEAN	98.1	---	---
	SD	1.8	---	---
	N	36	---	---
FREEZE-THAW-WASH RECOVERY (%)	MEAN	---	87.9	---
	SD	---	3.6	---
	N	---	36	---
HEMATOCRIT (V%)	MEAN	60	50	48
	SD	7	4	4
	N	36	36	35
HEMOGLOBIN (gm/dl)	MEAN	17.8	13.6	13.4
	SD	2.2	1.4	1.3
	N	36	36	35
RBC COUNT ($X10^9$/ml)	MEAN	6.1	4.6	4.5
	SD	0.6	0.4	0.4
	N	36	36	34
WBC COUNT ($X10^6$/ml)	MEAN	1.9	<0.5	---
	SD	0.9	0	---
	N	36	35	---
MANUAL WBC COUNT (#/ul)	MEAN	---	---	12.4
	SD	---	---	12.6
	N	---	---	9
TOTAL WBC ($X10^6$ /UNIT)	MEAN	513	---	3.7
	SD	261	---	3.8
	N	36	---	9
PLATELET COUNT ($X10^6$/ml)	MEAN	42	1.3	1.0
	SD	21	3.0	1.6
	N	36	35	29
BLOOD pH AT 22 C	MEAN	6.9	6.4	6.3
	SD	0.1	0.1	0.1
	N	27	33	33
SUPERNATANT OSMOLALITY (mOsm/kg H_2O)	MEAN	4662	299	302
	SD	267	6	5
	N	36	36	33
SUPERNATANT K+ (mEq/l)	MEAN	16.3	1.1	22.8
	SD	4.5	0.7	5.7
	N	36	36	35
SUPERNATANT HB (mg/dl)	MEAN	883	41	143
	SD	793	24	61
	N	36	36	35
% HEMOLYSIS	MEAN	---	0.2	0.5
	SD	---	0.1	0.2
	N	---	36	35

TABLE 3

RED BLOOD CELLS COLLECTED INTO CPD/AS-1 AND STORED AT 4 C FOR 42 DAYS AT NBRL, BOSTON, MA

		DAY 42
HEMATOCRIT (V%)	MEAN	53
	SD	2
	N	10
HEMOGLOBIN (gm/dl)	MEAN	17.0
	SD	0.9
	N	10
RBC COUNT ($X10^9$/ml)	MEAN	5.7
	SD	0.2
	N	10
WBC COUNT ($X10^6$/ml)	MEAN	3.2
	SD	1.4
	N	10
TOTAL WBC ($X\ 10^9$ PER UNIT)	MEAN	1.1
	SD	0.5
	N	10
PLATELET COUNT ($X10^6$/ml)	MEAN	51
	SD	29
	N	10
BLOOD pH AT 22 C	MEAN	6.5
	SD	0.1
	N	9
SUPERNATANT HB (mg/dl)	MEAN	203
	SD	67
	N	8
% HEMOLYSIS	MEAN	0.6
	SD	0.2
	N	8

TABLE 4

24-HOUR POSTTRANFSUSION SURVIVAL, LIFESPAN, AND INDEX OF THERAPEUTIC EFFECTIVENESS OF RED BLOOD CELLS DEGLYCEROLIZED IN THE HAEMONETICS MODEL 215 AND STORED AT 4 C IN AS-3 ADDITIVE SOLUTION FOR 15 DAYS

TEST SITE	LABEL METHOD		24-HOUR POSTTRANSFUSION SURVIVAL VALUE MEASURED USING A DOUBLE LABEL PROCEDURE (%)	LIFESPAN (T50, DAYS)	INDEX OF THERAPEUTIC EFFECTIVENESS* (%)
COMBINED		MEAN	77	28	68
		SD	9	4	8
		N	36	34	36
NBRL	51CR/125I ALBUMIN	MEAN	75	29	65
		SD	7	4	6
		N	14	13	14
WRAIR	51CR/99^{M}TC	MEAN	78	24	68
		SD	9	5	8
		N	8	7	8
UMMC	DOUBLE 51CR	MEAN	78	29	71
		SD	10	5	10
		N	14	14	14
CONTROL:	CPD/AS-1 RED BLOOD CELLS STORED AT 4 C FOR 42 DAYS				
NBRL		MEAN	72	27	72
		SD	5	3	5
		N	10	10	10

*PRODUCT OF IN VITRO FREEZE-THAW-WASH RECOVERY AND 24-HOUR POSTTRANSFUSION SURVIVAL VALUE

TABLE 5

COMPARISON OF 24-HOUR POSTTRANSFUSION SURVIVAL USING THE 51CR/125I ALBUMIN DOUBLE LABEL PROCEDURE OF AUTOLOGOUS RED BLOOD CELLS PROCESSED IN THE HAEMONETICS MODEL215 AND STORED AT 4 C IN AS-3 FOR 15 DAYS FOLLOWING DEGLYCEROLIZATION AND AUTOLOGOUS RED BLOOD CELLS COLLECTED INTO CPD/AS-1 AND STORED AT 4 C FOR 42 DAYS IN THE SAME DONOR AT THE NBRL, BOSTON, MA

DONOR	24-HOUR POSTTRANSFUSION SURVIVAL (%)	
	HAEMONETICS MODEL 215	CPD/AS-1
1	81	68
2	70	71
3	72	70
4	79	73
5	73	68
6	83	78
7	84	72
8	68	67
9	79	74
10	66	82
MEAN	76	72
SD	6	5
N	10	10

Paired T-test: Not significant, T=1.3

Appendix 3 (Pages 79-84)

Title: A Hypotonic Storage Solution Did Not Prolong the Viability of Red Blood Cells.

Authors: Babcock JG, Lippert LE, Derse-Anthony CP, Mechling M, and Hess JR.

A hypotonic storage solution did not prolong the viability of RBCs

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BACKGROUND: Hypotonic storage solutions and WBC filtration are both reported to improve RBC viability. This study tested the ability of an investigational hypotonic storage solution (AS-24, Medsep Corp.) to extend the viability of liquid-stored RBCs to 8 weeks.

STUDY DESIGN AND METHODS: In a pair of crossover trials, 11 RBC units, WBC-reduced by filtration and stored in AS-24 for 8 weeks, were compared with units from the same donors that were stored for 6 weeks in AS-3, and 13 RBC units, WBC-reduced by filtration and stored in AS-3 for 8 weeks, were compared with units from the same donors that were stored for 6 weeks in AS-3. Viability was measured by the $^{51}Cr/^{99m}Tc$ double-isotope method.

RESULTS: RBC viability at 8 weeks averaged 64 ± 3 percent in the AS-24 units and 67 ± 2 percent in the AS-3 units. It was equal at 77 ± 3 percent and 77 ± 2 percent after 6 weeks' storage in AS-3 in both trials.

CONCLUSIONS: Prestorage WBC reduction and storage in AS-24 did not extend RBC viability to 8 weeks. The improved viability previously demonstrated with storage of dilute suspensions of RBCs in hypotonic solutions is probably caused by factors other than the hypotonicity.

ABBREVIATIONS: MCV = mean corpuscular volume; RT = room temperature.

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Extending the shelf life of liquid-stored RBCs from 6 weeks to 8 weeks has the potential to increase the availability of blood in remote areas and improve the utility of autologous blood storage for elective surgery.[1] Ideally, an RBC component with an extended shelf life should be physiologically compatible with transfusion in large volumes, should retain conventional values of storage Hct and volume, and should conform to standard manufacturing practices.

In 1986, Meryman et al.[2] suggested that increasing the membrane surface tension of stored RBCs would limit membrane loss by reducing the budding of microvesicles from surface spicules that develop on preserved cells. They reported that hypotonic solutions that induced RBC swelling in vitro could be used to store RBCs for more than 14 weeks with greater than 75 percent viability. Additional reports have corroborated this finding.[3-9] However, those reports have two serious limitations. First, none of the studies examined the effect of hypotonic solutions alone in a system with a standard volume, a conventional Hct, or salts compatible with transfusion. Second, most of these studies relied on surrogates for viability as endpoints, such as RBC morphology and RBC ATP concentration.

The two studies reported here were undertaken to determine the ability of a hypotonic storage solution (AS-24, Medsep Corp., Covina, CA) to extend the viability of RBCs to 8 weeks. AS-24 is a hypotonic solution of the type described by Meryman et al.[2] and was manufactured under a license of their patent. The ability to extend viability was measured directly in a randomized crossover study comparing the viability of RBCs stored in AS-24 for 8 weeks with that of RBCs from the same donors that were stored in an isotonic commercial AS (AS-3, Nutricel, Medsep Corp.) for 6 weeks. In a parallel study, the viability of RBCs stored in AS-3 for 8 weeks was compared with that of RBCs from the same donors that were stored in AS-3 for 6 weeks. In each crossover study, the RBCs in the 8-week arm were WBC-reduced by filtration in an attempt to further improve viability,[10] whereas the RBCs in the 6-week arm were not WBC-reduced, so as to provide a conventionally stored standard. Viability was measured by the $^{51}Cr/^{99m}Tc$ double-isotope

technique 24 hours after the donor received, by IV infusion, a labeled sample from the RBC unit.

MATERIALS AND METHODS

Volunteers

Thirty healthy volunteers meeting standard blood donor criteria[11] were enrolled in one study or both studies after giving informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the US Army's Human Subjects Research Review Board. There were 30 subjects in all, with 2 subjects participating in both studies. Volunteers were tested to exclude sickle Hb trait (Sicklescreen, Pacific Hemostasis, Huntersville, NC) and abnormal osmotic fragility (Unopette Test 5830, Becton Dickinson, Rutherford, NJ).

Storage solutions

The compositions of AS-3 and AS-24 are shown in Table 1. The significant differences in the solutions are the presence of mannitol and the absence of sodium chloride in the AS-24 and vice versa in the AS-3. Both solutions were formulated to have a pH of 5.8 and were used as 100-mL ASs for packed RBCs collected into CP2D.

Study design

We conducted two parallel, randomized, crossover studies. Each study allowed the direct comparison of the viability of RBCs stored for 8 weeks and that of cells from the same donor stored for 6 weeks in a standard, licensed AS. The results of the two studies were then compared. In the first crossover study, RBC units were WBC-reduced by filtration and stored in AS-24 for 8 weeks. They were compared with non-WBC-reduced units from the same donors that were stored for 6 weeks in AS-3. In a second trial, RBC units were WBC-reduced by filtration and stored in AS-3 for 8 weeks. They were compared with second units from the same donor that were stored for 6 weeks in AS-3. Electrolytes, pH, glucose, lactate, RBC morphology scores, and RBC ATP concentrations were measured at the beginning and the end of storage. Viability was measured by the $^{51}Cr/^{99m}Tc$ double-isotope method.[12] Postinfusion survival of the RBCs was measured as the RBC ^{51}Cr retention, corrected for radioactive decay and 1 percent per day elution.

TABLE 1. Components of AS-24 and AS-3 in m*M* and solution pH

	AS-24	AS-3
Adenine	2	2
Dextrose	68.8	55.5
Mannitol	13.8	0
Na_3 citrate	17.7	20
NaH_2PO_4	13	23
NaCl	0	70.1
H_3 citrate	3	2
pH at RT	5.8	5.8

RBC unit preparation

Units of blood (450 ± 45 mL) were collected in CP2D and held for 7 hours at room temperature (RT).

AS-24 units (WBC-reduced) held for 8 weeks. Packed RBCs were prepared by centrifugation (5000 × *g*, 5 min, RT) followed by the removal of plasma to achieve a target storage Hct of 65 percent. AS-24 (100 mL) was added, and this step was followed by RT filtration to remove WBCs by using an integral filter (Leukotrap RC system, Medsep Corp.) and gravity flow.

AS-3 units (WBC-reduced) held for 8 weeks. Whole blood was filtered at RT to remove WBCs by using an integral filter (Leukotrap) and gravity flow. Packed RBCs were prepared by centrifugation (5000 × *g*, 5 min, RT), and this step was followed by removal of the plasma. Finally, 100 mL of AS-3 was added.

AS-3 units (non-WBC-reduced) held for 6 weeks. Packed RBCs were prepared by centrifugation (5000 × *g*, 5 min, RT), and this step was followed by the removal of plasma to achieve a target storage Hct of 65 percent. Finally, 100 mL of AS-3 was added.

All units were gently mixed, sampled for in vitro testing, and placed in refrigerated storage (1-6°C) 8 hours or less after collection.

In vitro measurements

Samples from stored units were collected into a small pouch attached via a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD) to the remaining donor needle tubing. A battery of in vitro tests was performed on all units immediately before and after storage. Filtered units were also sampled before filtration.

Total Hb concentration, unfiltered WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically (DU-62, Beckman, Fullerton, CA) using the modified Drabkin's assay.[13] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. Centrifuged microhematocrits (Clay Adams, Becton Dickinson) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzer. Mean corpuscular volume (MCV) was calculated from the centrifuged microhematocrit and the RBC count. Postfiltration WBC counts were taken by using a Neubauer hemocytometer and propidium iodide staining.[14] The RBC morphology score was determined according to the method of Usry et al.[15]

RBC ATP concentration was measured in deproteinized supernatants. Whole-blood or packed RBC aliquots were mixed with cold, 12-percent perchloric acid to precipi-

tate blood proteins and centrifuged at 2700 × *g* for 10 minutes; then the protein-free supernatant was adjusted to pH 8 to 9 with solid $KHCO_3$ and frozen at –80°C until tested. ATP was assayed enzymatically by the use of a commercially available kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Check for bacterial contamination

Three or 4 days before the end of storage, sterilely collected aliquots from each unit were tested for bacterial contamination in broth and agar cultures by using a commercial blood culture system (BBL Septi-Check, Becton Dickinson Microbiology Systems, Cockeysville, MD). Absence of bacteria growth in the incubated cultures after 72 hours was required before a sample from the unit was prepared for infusion to the donor.

In vivo RBC recovery and survival measurement

After 6 or 8 weeks of storage, in vivo RBC recovery was measured 24 hours after autologous infusion by using a double-radiolabel procedure. In brief, a sample of the stored blood was labeled with 20 µCi of ^{51}Cr. Concurrently, a fresh blood sample was collected from the volunteer and labeled with 25 µCi of ^{99m}Tc. Carefully measured aliquots of the radiolabeled RBCs were mixed and rapidly infused. Blood samples were collected at timed intervals during the 30 minutes immediately after the infusion and again at 24 hours, 7 days, and 14 days after infusion. The radioactivity of the samples was measured with a gamma counter (CliniGamma Counter, Model 1272, LKB, Turku, Finland).

Gamma emissions from ^{99m}Tc-radiolabeled cells were measured in the samples collected during the 30 minutes after infusion and used to determine an independent RBC volume. The activity from ^{51}Cr-labeled cells was measured in all of the samples and used to calculate the fractional survival of the stored RBCs.

Statistical analysis

Comparisons of means within the individual crossover trials were made with the paired *t* test. For comparisons between the crossover trials, the unpaired *t* test was used. The correlations between the morphology scores and viability and the RBC ATP concentrations and viability were performed by the Pearson method. Probabilities less than 0.05 were considered significant.

RESULTS

Volunteers

Eleven volunteers completed the first study, and 13 completed the second study. Two individuals participated in and completed both studies. The remaining 8 volunteers withdrew for a variety of reasons; in four cases, the reason(s) was related to the study. Two donors withdrew after unsuccessful phlebotomy, 1 after a tubing weld failure contaminated a unit, and 1 after infiltration of an infusion site.

Prestorage equivalence of groups

Initial characteristics of the blood units are presented in Table 2. The units contained an average of 206 ± 12 mL of RBCs at collection. WBC-reduced units lost approximately 6 percent of their RBCs in the filter, and 14 mL of RBCs was removed from all units for prestorage testing. An additional 4 mL was removed from each WBC-reduced unit to allow postfiltration WBC counting. Plasma was removed from the units to produce packed RBCs with a storage Hct of 65 percent. The units began storage with RBC ATP concentrations averaging 5.1 ± 0.2 µmol per g of Hb and negligible hemolysis, which were equal in all groups.

Hypotonic swelling

As indicated by a 14-percent increase in MCV, from 95.7 ± 4.0 fL to 109.5 ± 6.7 fL (shown in Table 2), the AS-24 solution produced significant cell swelling. However, AS-3, which is isotonic with an effective osmolality of 270 mOsm, also induced an increase in the MCV of the other units, which averaged 8 fL. During the storage period, the AS-24 cells showed the greatest loss of volume, about 4 percent.

WBC filtration

In the AS-24 units, the WBC-reduction filter reduced the WBC count from 6082 per µL to 0.63 per µL, a 4 log reduction, to an average of 2.3×10^5 WBCs per unit. The average time needed to filter the AS-24 units was 40 minutes, because of the higher Hct of the RBC component and the size of the swollen cells. The average filtration time for anticoagulated whole blood in the AS-3 units was 9 minutes, and the filter reduced the WBC numbers below the detection limits of our assay (0.1/µL or 3.5×10^4/unit). Despite the different preparative stages at which the AS-24 and AS-3

TABLE 2. Similarity of stored unit characteristics at the beginning of storage*

	First study (n = 11)		Second study (n = 13)	
	8 weeks, filtered, in AS-24	6 weeks, unfiltered, in AS-3	8 weeks, filtered, in AS-3	6 weeks, unfiltered, in AS-3
Hct (%)	64.9 ± 4.0	62.5 ± 3.1	68.4 ± 5.0	67.8 ± 1.9
MCV (fL)	109.5 ± 6.7†	101.2 ± 4.6	103.2 ± 7.0	101.3 ± 3.2
ATP (µmol/g Hb)	5.1 ± 0.3	5.1 ± 0.2	5.0 ± 0.5	5.2 ± 0.7
Hemolysis (%)	0.026 ± 0.020	0.016 ± 0.022	0.014 ± 0.030	0.012 ± 0.021
Morphology index	93.4 ± 3.1	93.5 ± 3.6	89.2 ± 4.0	88.7 ± 3.5

* Data presented as mean ± SD.
† Only the MCV in these AS-24 units was significantly greater than that in its paired control, as a result of the hypotonic solution.

units were filtered, both groups lost only 6 percent of all RBCs (23 g of concentrated AS-24 product vs. 35 g of whole blood for the AS-3 units). Both groups achieved WBC-reduction standards established in previous validation of the filters.

Poststorage hemolysis, RBC ATP concentrations, and RBC morphology

Hemolysis averaged less than 1 percent under all conditions of storage in both studies (Table 3). WBC-reduced units in each study had significantly less hemolysis, despite 2-week longer storage. RBC ATP concentrations were lower in the units stored for 8 weeks in both studies than those in cells from the same donors that were stored for 6 weeks in AS-3. Despite the slight baseline differences in the two crossover studies and 2 additional weeks of storage for the 8-week units, there was no significant difference in the morphology scores of the four groups after storage.

Poststorage RBC viability

The 24-hour recovery fraction measures for the two studies are illustrated in Fig. 1. The mean 24-hour survivals for the 6-week AS-3 "control" arm of each study were equivalent at 77 percent and greater than the FDA standard of 75 percent. The 8-week arms of each trial had significantly lower survivals, at 64 ± 3 percent for the AS-24 units and 67 ± 2 percent for the AS-3 units. For all units, about 80 percent of the RBCs surviving 24 hours persisted in the circulation for 14 days.

The relationship of RBC morphology score and RBC ATP concentration to RBC viability

An analysis of the relationship between the RBC morphology score and viability showed a significant correlation, $p = 0.001$. However, the correlation explained only 21 percent of the relationship (Fig. 2). No morphology score defined a clear threshold for predicting adequate in vivo survival. RBC morphology scores of 60 or greater were counted for 9 of the 48 units; yet, for 4 of these, the 24-hour survival values were below 75 percent. Likewise, of 19 units for which viability studies met or exceeded 75-percent recovery, morphology scores for 12 were below 60. The stored blood unit with the highest 24-hour recovery fraction (95%) had a morphology score of only 55.

The correlation between RBC ATP concentration and RBC viability was stronger, accounting for 40 percent of the variability in the relationship (Fig. 3). A decrease of 1 µmol per g of Hb in the RBC ATP concentration at the end of storage is expected to reduce viability by 8.3 ± 1.5 percent, according to the observed regression coefficient. However, the positive predictive value of an RBC ATP concentration of 2 µmol per g of Hb or greater was weak. Only 19 of the 41 units with RBC ATP concentrations meeting this criterion exhibited viability of 75 percent or greater, but all 7 units below that threshold demonstrated unacceptably low viability. Only 12 of 17 units with an RBC ATP concentration equal to or greater than 3 µmol per g of Hb had a 24-hour survival of 75 percent.

TABLE 3. Differences in stored unit characteristics at the end of storage*

	First study (n = 11)		Second study (n = 13)	
	8 weeks, filtered, in AS-24	6 weeks, unfiltered, in AS-3	8 weeks, filtered, in AS-3	6 weeks, unfiltered, in AS-3
MCV (fL)	105.6 ± 4.8†	100.1 ± 5.3	100.9 ± 4.2	100.3 ± 3.7
ATP (µmol/g Hb)	2.0 ± 0.5†	3.1 ± 0.6	2.5 ± 0.5†	3.2 ± 0.6
Hemolysis (%)	0.31 ± 0.15†	0.49 ± 0.31	0.20 ± 0.07†	0.51 ± 0.39
Morphology index	53.7 ± 4.5	55.8 ± 5.3	52.6 ± 4.6†	59.9 ± 7.2

* Data presented as mean ± SD.
† Significantly different from their paired controls at the $p<0.05$ level. The lower hemolysis in the filtered units is probably clinically important, as it occurred despite the units being stored for 2 weeks longer and having lower RBC ATP concentrations and recovery.

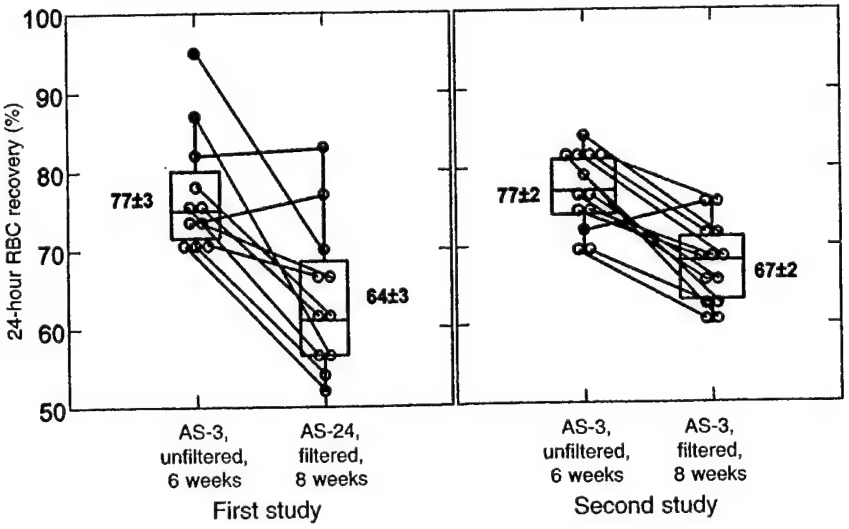

Fig. 1. The distribution of RBC recovery measures in the two studies. The paired measures are connected and the mean values are presented as mean ± SD.

DISCUSSION

In this investigation, we set out to test the prediction that a hypotonic AS (AS-24) would increase the viability of stored RBCs. Our paired crossover study design produced initially equivalent groups, and AS-24 did produce hypotonic swelling of the stored RBCs. Nevertheless, the viability was not usefully prolonged. Viability after 8 weeks' storage in AS-24

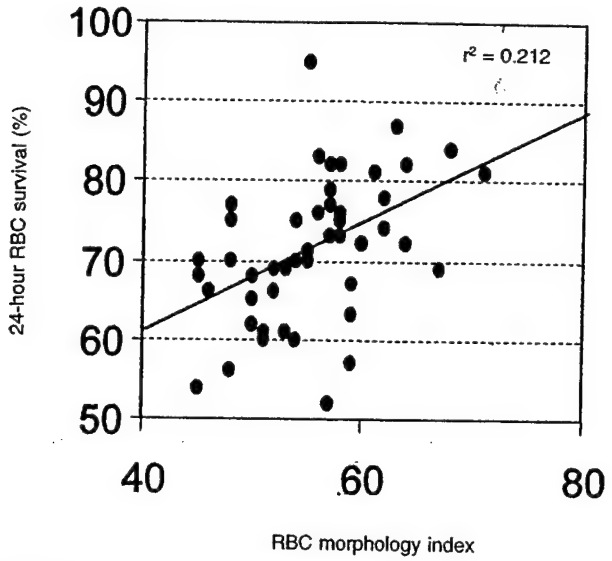

Fig. 2. The relationship of the RBC morphology index to the in vivo RBC recovery fraction. The prestorage morphology scores are very similar in all arms of the study and show no effect from storage in either solution to the recovery point.

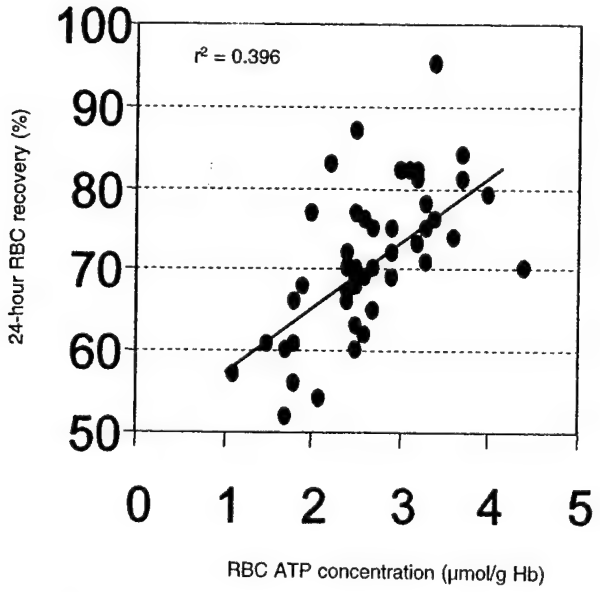

Fig. 3. The relationship of the RBC ATP concentration to the in vivo RBC recovery fraction. The RBC ATP concentration demonstrates better correlation with in vitro RBC recovery than with in vivo recovery (see Fig. 2).

appeared to be no better than that after 8 weeks' storage in AS-3 when the RBCs were stored under otherwise equivalent conditions. WBC-reduction by filtration decreased RBC hemolysis by one-half during storage in the 8-week arms of both trials, despite the additional 2 weeks of storage. There appeared to be no additional effect on reducing hemolysis conferred by storage in AS-24. Further, the RBC morphology scores of the WBC-reduced units stored for 8 weeks were equal in the trials and equivalent to those of the non-WBC-reduced units after 6 weeks of storage. In sum, the studies described here provide no evidence that the hypotonic AS (AS-24) improved viability, increased RBC morphology scores, or reduced hemolysis.

The 24-hour recovery of the RBCs stored for 6 weeks in AS-3 is lower than that in some previous reports,[10,16] but it is not significantly different from that reported by Arduini et al.[17] This lower-than-usual recovery appears to represent a particularly low subset within the range of viability measurements to be expected with a randomly chosen group of volunteers. The decrease in RBC ATP concentration with time between the endpoints of the 6- and 8-week arms was about 0.4 µmol per g of Hb per week, which is typical of the decrease seen in other storage studies with repeated sampling of RBC units.[18] The decrease in measured viability of 10 to 13 percent between 6 and 8 weeks is approximately what would be predicted from the decrease in RBC ATP concentration. The results suggest that AS-24 is no better than AS-3 when used in the 100-mL AS format.

In their original description of hypotonic ASs, Meryman et al.[2] showed that RBCs, stored at a Hct of 35 to 40 percent with an initial pH of 7.1 in a hypotonic solution containing high concentrations of ammonium, potassium, phosphate, and membrane protectants such as mannitol and citrate, contained viable cells for as long as 19 weeks. The report was important, because it showed that such extended storage was possible. The most successful of their solutions, "Solution 6," caused RBC ATP concentrations to rise to 160 percent of starting values at 4 weeks of storage. RBC ATP concentrations declined steadily thereafter, at about 10 percent of the starting value (0.4 µmol/g Hb) each week.

Four groups have subsequently reexamined this concept. In 1990, Greenwalt and colleagues[5] confirmed the increased RBC ATP concentrations with storage in Solution 6 in split-unit studies. Although they could also confirm better RBC morphology scores with storage in the hypotonic media than with storage in AS-1, they could not confirm RBC swelling by optical methods, and the loss of Hb in microvesicles was the same under both conditions of storage.[19,20] In another 1990 report, Mazor et al.[6] replaced the potassium and ammonium in Solution 6 with sodium and with rubidium, another monovalent cation, and found no difference in the initial rise in RBC ATP concentrations. In a 1994 report,[7] the same group found that hypotonicity did not affect the RBC ATP concentration, but that initial pH, phosphate concentration, and adenine concentration were important. This group performed no studies of RBC viability. In 1992, Kay and Beutler[8] also confirmed the ability of Solution 6 to increase RBC ATP concentrations. They concluded that ammonium was important because it disinhibited phosphofructokinase. However, they could not

demonstrate improved viability with the storage of rabbit RBCs in Solution 6. Finally, in 1996, Dumaswala and colleagues[9] reported a comparison of two hypotonic media, one with phosphate and one without. The less hypotonic medium with and a higher initial pH had better preservation of RBC ATP concentrations.

The demonstration of 14-week RBC storage by Meryman et al. has always been clouded by the recognition that, "[f]rom the clinical standpoint, Solution number 6 would clearly be unacceptable for transfusion...."[2(p505)] Attempts to understand and translate this work into clinically useful storage solutions have been limited by the failure to measure clinically relevant endpoints, such as human RBC viability and storage hemolysis, with reasonable statistical power. Future work in this field should attempt to maximize RBC ATP concentrations by the manipulation of pH, adenine, and phosphate content and to minimize hemolysis by WBC-reduction. Progress can be made with candidate ASs and storage systems incorporating these innovations.

ACKNOWLEDGMENT

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REFERENCES

1. Hess JR. Extended liquid storage of red blood cells. In: Manning FJ, Sparacino L, eds. Blood donors and the supply of blood and blood products. Washington: National Academy Press, 1996:99-102.
2. Meryman HT, Hornblower ML, Syring RL. Prolonged storage of red cells at 4°C. Transfusion 1986:26:500-5.
3. Meryman HT, Hornblower ML, Syring RL, Mesbah-Karimi N. Extending the storage of red cells at 4°C. Transfus Sci 1994:15:105-15.
4. Meryman HT, Hornblower ML, Keegan T, et al. Refrigerated storage of washed red cells. Vox Sang 1991:60:88-98.
5. Greenwalt TJ, McGuinness CG, Dumaswala UJ, Carter HW. Studies in red blood cell preservation. 3. A phosphate-ammonium-adenine additive solution. Vox Sang 1990;58:94-9.
6. Mazor D, Dvilansky A, Meyerstein N. Prolonged storage of red cells with ammonium chloride and mannitol. Transfusion 1990;30:150-3.
7. Mazor D, Dvilansky A, Meyerstein N. Prolonged storage of red cells: the effect of pH, adenine and phosphate. Vox Sang 1994;66:264-9.
8. Kay A, Beutler E. The effect of ammonium, phosphate, potassium, and hypotonicity on stored red cells. Transfusion 1992;32:37-41.
9. Dumaswala UJ, Wilson MJ, José T, Daleke DL. Glutamine- and phosphate-containing hypotonic storage media better maintain erythrocyte membrane physical properties. Blood 1996;88:697-704.
10. Heaton WA, Holme S, Smith K, et al. Effects of 3-5 $\log_{10}$ prestorage leucocyte depletion on red cell storage and metabolism. Br J Haematol 1994;87:363-8.
11. Vengelen-Tyler V, ed. Technical manual. 12th ed. Bethesda: American Association of Blood Banks, 1996.
12. Davey RJ. Red cell radiolabeling in transfusion medicine. In: Davey RJ, Wallace ME, eds. Diagnostic and investigational uses of radiolabeled blood elements. Arlington: American Association of Blood Banks, 1987:39-66.
13. Moore GL, Ledford ME, Merydith A. A micromodification of the Drabkin Hb assay for measuring plasma Hb in the range of 5 to 2000 mg/dl. Biochem Med 1981;26:167-73.
14. Kao KJ, Scornick JC. Accurate quantitation of low numbers of white cells in white cell-depleted blood components. Transfusion 1989;29:774-7.
15. Usry RT, Moore GL, Manalo FW. Morphology of stored, rejuvenated human erythrocytes. Vox Sang 1975;28:176-83.
16. Simon TL, Marcus CS, Myhre BA, Nelson EJ. Effects of AS-3 nutrient-additive solution on 42 and 49 days of storage of red cells. Transfusion 1987;27:178-82.
17. Arduini A, Holme S, Sweeney JD, et al. Addition of L-carnitine to additive solution-suspended red cells stored at 4°C reduces in vitro hemolysis and improves in vivo viability. Transfusion 1997;37:166-74.
18. Dumaswala UJ, Oreskovic RT, Petrosky TL, Greenwalt TJ. Studies in red blood cell preservation; 5. Determining the limiting concentrations of NH_4Cl and Na_2HPO_4 needed to maintain red blood cell ATP during storage. Vox Sang 1992;62:136-40.
19. Greenwalt TJ, Rugg N, Dumaswala UJ. The effect of hypotonicity, glutamine, and glycine on red cell preservation. Transfusion 1997;37:269-76.
20. Greenwalt TJ, Dumaswala UJ, Dhringa N, et al. Studies in red blood cell preservation. 7. In vivo and in vitro studies with a modified phosphate-ammonium additive solution. Vox Sang 1993;65:87-94.

Appendix 4 (Pages 86-92)

Title: The Effects of Phosphate, pH, and AS Volume on RBCs Stored in Saline-Adenine-Glucose-Mannitol Solutions.

Authors: Hess JR, Lippert LE, Derse-Anthony CP, Hill HR, Oliver CK, Rugg N, Knapp AD, Gormas JF, and Greenwalt TJ.

The effects of phosphate, pH, and AS volume on RBCs stored in saline-adenine-glucose-mannitol solutions

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BACKGROUND: RBC ATP concentrations are the most important correlate of RBC viability. Tests were performed to determine whether increased AS volume, pH, and phosphate content increased stored RBC ATP concentrations.

STUDY DESIGN AND METHODS: In three studies, packed RBCs were pooled in groups of 3 or 4 units and realiquoted as combined units to reduce intradonor differences. Pooled units were stored in the licensed ASs, AS-1 or AS-5, which contain saline, adenine, glucose, and mannitol (SAGM), or in experimental ASs (EASs) containing SAGM and disodium phosphate. Ten pools were stored in AS-1 at RBC concentrations equivalent to 100, 200, or 300 mL of AS. Six pools were stored in 100, 200, 300, or 400 mL volumes of EAS-61. Ten pools were stored in 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64. RBC ATP concentration and other measures of RBC metabolism and function were measured weekly.

RESULTS: RBC ATP concentrations decreased sooner with storage in increasing volumes of AS-1. In EAS-61 and EAS-64, RBC ATP concentrations initially increased and stayed elevated longer with increasing AS volume.

CONCLUSIONS: The addition of disodium phosphate to SAGM AS increases the RBC ATP concentrations. Reducing storage Hct appears to have a separate beneficial effect in reducing hemolysis.

RBC storage systems with longer outdating will have many beneficial effects.[1] The burden of nonviable RBCs will be reduced for most transfusion recipients. Some of the several hundred thousand RBCs that now outdate each year will not be lost, and the ability to maintain adequate emergency stocks in remote locations will be enhanced. Autologous transfusion schemes will function better if RBCs can be stored longer, have better viability, and contain fewer products of cell degradation.

The development of better RBC storage systems has been slow. For the last decade, 6-week RBC storage has been the United States standard. That standard is based on the use of 100-mL of AS containing either saline, adenine, glucose, and mannitol (SAGM, as in AS-1 and AS-5) or saline, adenine, glucose, and monosodium phosphate (SAGP, as in AS-3).[2] Two 7-week RBC storage systems have been licensed in Europe,[3,4] but the 24-hour viability of cells stored in these solutions is barely 75 percent. The promise of 14-week RBC

ABBREVIATIONS: EAS(s) = experimental AS(s); SAGM = saline-adenine-glucose-mannitol.

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DISCLOSURE: The authors certify that they are employed, directly or indirectly, by the U.S. Army and the University of Cincinnati, which have jointly filed for patent rights on EAS-61 and EAS-64. Two of the authors (JRH and TJG) are listed as inventors on the patent filing.

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storage heralded in a 1986 article by Meryman et al.[5] has not been realized in transfusable solutions. Nevertheless, the fact that long-term viability is possible in solutions that drive ATP synthesis suggests the most likely path for future development.

We report here the results of three pooling studies that measured RBC ATP concentrations with increasing volumes of the ASs. The experimental ASs (EASs) with greater phosphate, pH, and volume increased RBC ATP concentrations, whereas the traditional SAGM solutions led to decreased RBC ATP concentrations at greater dilutions. These studies were performed in two separate laboratories. They are reported together because they help explain why present storage solutions limit viability to 6 weeks and how transfusable solutions that will extend RBC viability beyond that limit might be formulated.

MATERIALS AND METHODS

Volunteers

Two groups of 30 healthy volunteers, who were donating full units of blood for the collection of anthrax-immune plasma, consented to the use of their RBCs for Studies 1 and 3. The donors met standard blood donor criteria and gave informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the US Army's Human Subjects Research Review Board. Volunteers were tested to exclude sickle Hb trait (Sicklescreen, Pacific Hemostasis, Huntersville, NC).

In Study 2, 24 volunteers donated full units of blood under protocols approved by the Hoxworth Blood Center and the University of Cincinnati's Institutional Review Board.

Storage solutions

The compositions of AS-1 (Adsol, Baxter Healthcare Corp., Roundlake, IL), AS-5 (Optisol, Terumo Medical Corp., Somerset, NJ), EAS-61, and EAS-64 are compared in Table 1. The significant differences in the solutions are the reduced salt content and the presence of disodium phosphate in the EASs. The presence of disodium phosphate raises the pH of the EASs to about 8.5 at room temperature, whereas the pH of AS-1 and AS-5 is 5.5.

The EASs were made in the laboratory from high-purity adenine, sugars, and salts and were sterilely filtered into 1-L storage bags. When sterility was confirmed by the absence of bacterial and fungal growth for 7 to 14 days, the solutions were aliquoted by weight into 600-mL bags. All connections were made by using a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD).

Study design

We conducted three pooling studies to evaluate RBC metabolism and physiology over the period of storage. Pooling reduces the largest source of variability in conventional blood storage studies—the differences between the RBCs from different donors—by placing some of the cells from every donor in every arm of the study while maintaining conventional unit size and geometry. In each of the three studies, the RBC units were grouped into sets of 3 or 4 ABO-matched units; each set was then pooled, mixed, and realiquoted into identical pooled units. For example, in Study 1, 30 RBC units were collected and grouped into 10 sets of 3 ABO-matched units. Each set of 3 matched units was then pooled, mixed, and realiquoted as 3 identical pooled units. Ten pooled units, 1 from each set, were used in each arm of the study.

TABLE 1. Composition of ASs used in this study (concentrations in m*M*)

	CPD	AS-1	AS-5	EAS-61	EAS-64
Dextrose	142	111	45	110	50
Na_3 citrate	104				
Adenine		2	2.2	2	2
NaCl		154	150	26	75
Mannitol		41	45.5	55	20
Citric acid	18				
Na_2HPO_4				12	9
pH at room temperature	5.5	5.5	5.5	8.2	8.5

In Study 1, units were stored in AS-1 at RBC concentrations equivalent to dilution in 100, 200, and 300 mL of AS. In Study 2, units were stored in 100-, 200-, 300-, or 400-mL volumes of EAS-61. In Study 3, units were stored in 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64. Electrolytes, pH, metabolites, RBC morphology scores, and RBC ATP concentrations were measured weekly during storage by sterile sampling techniques.

RBC unit preparation

Study 1, AS-1 units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of an AS-1 triple-bag collection set (code 4R-1436, Baxter). Packed cells were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 80 to 85 percent. ABO-matched packed RBCs were then pooled in groups of 3 units in 1-L sterile bags (Code 4R-20-32, Baxter), mixed thoroughly, and aliquoted into the study units (by weight) using a sterile connecting device for all the transfers. The study units were prepared in 600-mL transfer bags (Code 4R-2023, Baxter) by mixture of the packed RBC aliquot with sufficient AS to produce final storage Hcts of 55, 43, and 35 percent. The result is essentially equivalent to the addition of 100, 200, or 300 mL of AS, respectively, to 200 mL of RBCs.

Study 2, EAS-61 units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of an AS-1 triple-bag collection set (Code 4R-1436). Packed cells were prepared by centrifuga-

tion at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of plasma to achieve a target Hct of 80 percent. Packed RBCs were pooled, as above, in sets of 4 units and realiquoted. Finally, EAS-61 was added in amounts of 100, 200, 300, and 400 mL to achieve target storage Hcts of about 66, 55, 46, and 40 percent, respectively. The storage Hcts are higher with EAS-61 than with AS-1 because the hypotonic EAS leads to a 20-percent swelling of the RBCs with an equivalent increase in the cellular fractional volume.

Study 3, AS-5, EAS-61, and EAS-64 units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of an AS-5 double-bag collection set (Code 1BB*AGD456A, Terumo). Packed cells were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 80 to 85 percent. Three units of identical ABO type were then pooled and realiquoted as described above, and either 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64 was added. All units were gently mixed by inversion 25 times, sampled aseptically for in vitro testing, and placed in refrigerated storage (1-6°C) 4 hours or less after collection.

In vitro measurements

Samples from stored units were collected, after gentle mixing by inversion, by a sterile-sampling procedure in a biologic safety cabinet. A battery of in vitro tests was performed on all units at the beginning of storage and weekly thereafter.

For Studies 1 and 3, performed at Walter Reed, the total Hb concentration, WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically by using the modified Drabkin's assay.[6] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. The results are expressed as the percentage of hemolysis to compensate for the differences in Hct and Hb concentrations in samples. Centrifuged microHcts (Clay Adams, Becton Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzers. Mean corpuscular volume was calculated from the microHct and the RBC count.

RBC ATP concentrations were measured in deproteinized supernatants. Whole-blood or packed cell aliquots were mixed with cold 10-percent trichloroacetic acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until it was tested. ATP was assayed enzymatically by using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Blood gases and pH were measured on a blood gas analyzer (855, Corning, Ithaca, NY). Thus, pH was measured at 37°C. Phosphate and glucose were measured on a programmable chemical analyzer (Cobas Fara, Roche Diagnostic Systems, Nutley, NJ).

For Study 2, performed at the Hoxworth Blood Center, a different clinical hematology analyzer was used (MaxM, Coulter Electronics, Hialeah, FL). The pH was measured with a benchtop pH meter (900A Orion, Research, Boston, MA) at 22°C. Blood pH has a temperature coefficient of –0.015 pH units per degree (C), so pH measured at 22°C will be about 0.22 pH units higher than pH measured at 37°C. Supernatant potassium, glucose, and inorganic phosphorus testing was sent to an outside laboratory (Health Alliance Laboratories, Cincinnati, OH). Inorganic phosphate was measured by using a procedure of Boehringer Mannheim (Mannheim, Germany) that involves the formation of ammonium phosphomolybdate, which was detected in an automated chemistry analyzer (747-200, Boehringer Mannheim Corp., Indianapolis, IN). RBC ATP concentrations were measured as above, except that the RBC suspensions were deproteinized with 12-percent trichloroacetic acid. Supernatant Hb was measured with 3,3´,5,5´-tetramethylbenzidine (Procedure No. 527, Sigma Diagnostics). The average degree of RBC shape change from discocytes to echinocytes to spherocytes was measured as RBC morphology scores on 200 cells, determined according to the method of Usry et al.[7]

Statistical analysis

Comparisons of means of measured values at given times within the individual crossover trials were evaluated with ANOVA. Probabilities less than 0.05 were considered significant.

RESULTS

Study 1. RBCs were stored in increasing volumes of AS-1. RBCs stored in the conventional 100 mL of AS-1 maintained RBC ATP concentrations close to their initial value of 5.2 µmol per g of Hb for 4 weeks (Fig. 1A). Thereafter, the RBC ATP concentration declined by 0.6 µmol per g of Hb per week. With increasing volumes of the AS, the initial RBC ATP concentration rose higher, to 5.5 and 5.8 µmol per g of Hb, but the subsequent decline began sooner, at 2.5 and 1 weeks, respectively. However, the rate of decline remained the same at 0.6 µmol per g of Hb per week.

Supernatant pH was the same at each weekly measurement at all three suspension concentrations (Fig. 1B). Inorganic phosphate, initially from the residual plasma and the CPD and later from the breakdown of ATP and 2,3 DPG, was diluted in the additional volumes of AS-1 (Fig. 1C). The additional AS-1 volume decreased the RBC hemolysis during storage by approximately 50 percent (Fig. 1D).

Study 2. RBCs were stored in increasing volumes of EAS-61. RBC ATP concentrations were different after just 1 hour of storage, averaging 4.11 ± 0.37 µmol per g of Hb with 100 mL of EAS-61, 4.54 ± 0.34 with 200 mL, 4.96 ± 0.15 with 300

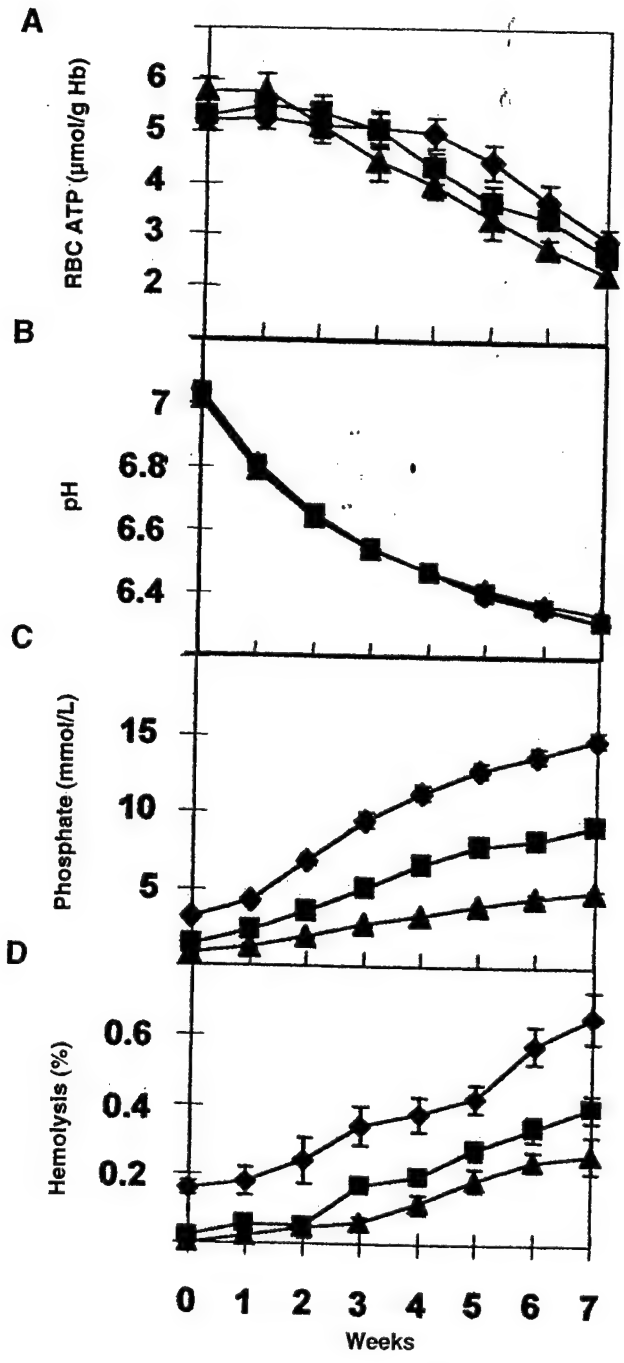

Fig. 1. The RBC ATP concentration (A), supernatant pH measured at 37°C (B), supernatant inorganic phosphate concentration (C), and fractional hemolysis (D) of pooled RBC units stored in the equivalent of 100 (-◆-), 200 (-■-), and 300 (-▲-) mL of AS-1 (n = 10 measurements at each point, data presented as mean ± SEM). The increasing volume of AS-1 led to reduced RBC ATP concentrations in the later phases of storage, despite equivalent pH and higher phosphate concentrations. The increased volume led to improved RBC integrity.

mL, and 5.11 ± 0.25 with 400 mL (Fig. 2A). Over the next 2 weeks, these concentrations increased to 4.38 ± 0.47, 4.88 ± 0.30, 5.34 ± 0.39, and 5.56 ± 0.39 µmol per g of Hb, respectively. After that time, the RBC ATP concentration declined at a uniform rate of about 0.35 µmol per g of Hb per week in all groups.

Supernatant pH was again the same at each weekly measurement (Fig. 2B). Inorganic phosphate concentrations were all approximately 6 mmol per dL (data not shown). The RBC morphology indices were 90- to 100-percent disco-cytes for all groups except for the 100-mL AS volume group (Fig. 2C). The AS volumes of 200 mL or greater were also associated with a 50-percent reduction in RBC hemolysis (Fig. 2D).

Study 3. RBCs were stored in 100 mL of AS-5, 200 mL of EAS-61, and 300 mL of EAS-64. RBC ATP concentrations were initially identical in all groups, about 4.8 ± 0.3 µmol per g of Hb. They remained stable in the AS-5 group for the next 2 weeks, but, in the EASs, they increased by about 10 percent over the first 2 weeks and remained elevated until 5 weeks (Fig. 3A). In AS-5, the RBC ATP concentrations decreased after 2 weeks of storage, at a steady rate of about 0.4 µmol per g of Hb per week. The same rate of decrease was observed with storage in the EASs, but the decrease started at 5 weeks of storage. Thus, the RBC ATP concentrations in the units stored in the EASs were about 1 µmol per g of Hb higher between 5 and 10 weeks of storage, or they remained above any given level for about 2.5 weeks longer.

Supernatant pH decreased more rapidly in the first 2 weeks in the smaller volume of the AS-5 system and less rapidly in the later phases of storage (Fig. 3B). Supernatant phosphate concentrations behaved very differently in the three storage solutions, rising continuously in AS-5, decreasing initially in EAS-61, and remaining relatively constant in EAS-64 (Fig. 3C). Both of the EASs reduced RBC hemolysis by 75 percent (Fig. 3D).

DISCUSSION

In 1986, Meryman et al.[5] showed that RBCs could be stored in experimental nutrient solutions for as long as 14 weeks and could have in vivo 24-hour recoveries greater than 75 percent. Their storage solutions included conventional ingredients such as adenine, phosphate, citrate, and mannitol, but also unconventional ingredients such as ammonium and potassium salts. They also used unconventional formulations with low salt concentrations and neutral pH. The group was not able to discover which of the ingredients were responsible for the marked elevation of RBC ATP concentrations that seemed to be responsible for the prolonged viability.

Five laboratories have confirmed and attempted to extend the work of Meryman's group. In 1990, Greenwalt and colleagues[8] reported splitting units of RBCs and storing them, half in AS-1 and half in Meryman's Solution 6.

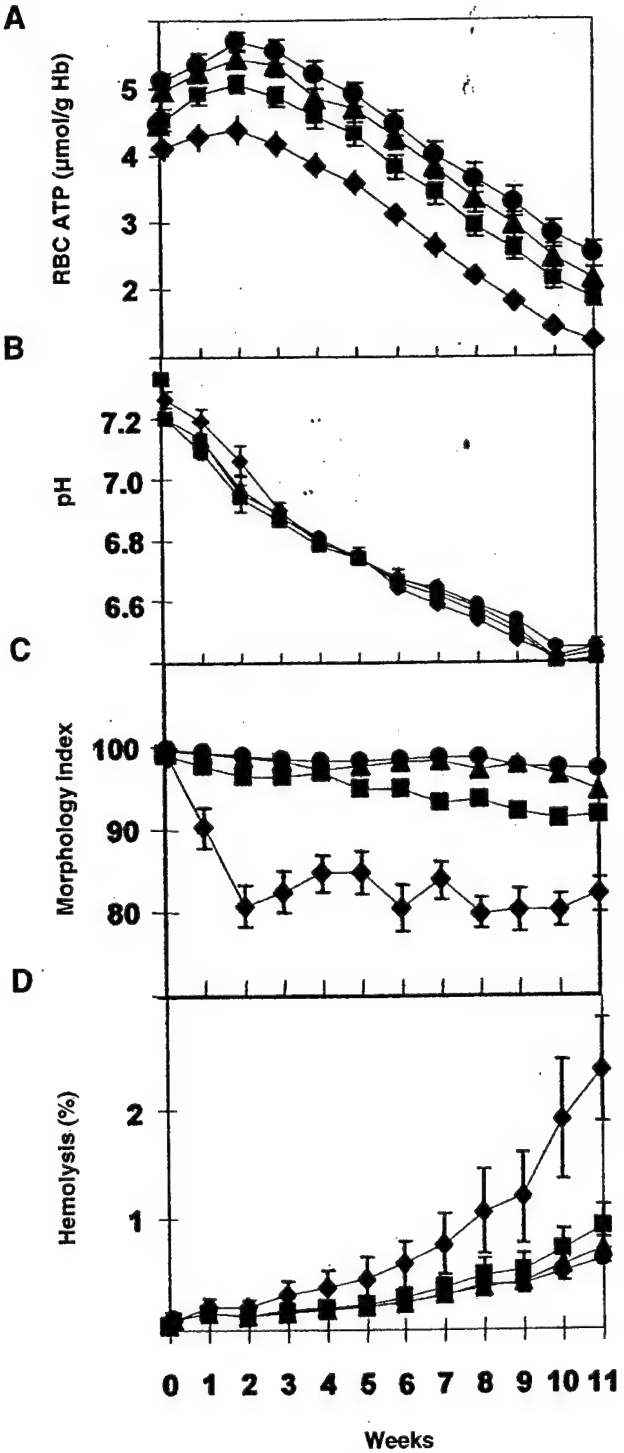

Fig. 2. The RBC ATP concentration (A), supernatant pH measured at 22°C (B),morphology index (C), and fractional hemolysis (D) of pooled RBC units stored in 100 (-◆-), 200 (-■-), 300 (-▲-), and 400 (-●-) mL of EAS-61 (n = 6 measurements at each point, data presented as mean ± SEM). The increasing volume of AS led to increased RBC ATP concentrations in the later phases of storage, despite equivalent pH. The increased volume led to improved morphology indexes and improved RBC integrity.

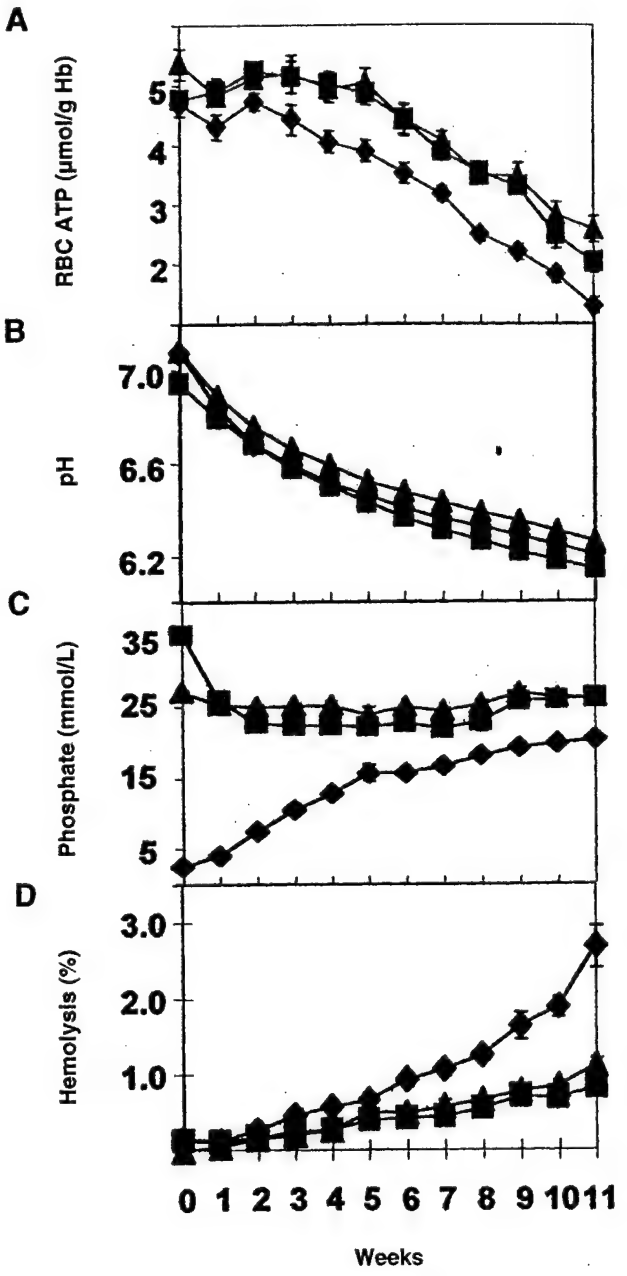

Fig. 3. The RBC ATP concentration (A), supernatant pH measured at 37°C (B), supernatant inorganic phosphate concentration (C), and fractional hemolysis (D) of pooled RBC units stored in 100 mL of AS-5 (-◆-), 200 mL of EAS-61 (-■-), and 300 mL of EAS-64 (-▲-) (n = 10 measurements at each point, data presented as mean ± SEM). The EASs appear to maintain RBC ATP concentrations 2.5 to 3 weeks longer and cause a marked reduction in RBC hemolysis during prolonged storage.

They were able to confirm the increased RBC ATP concentrations in the Meryman solution, but also showed that the low salt solution did not result in better membrane preservation. In 1992, this group reported that increasing amounts of disodium phosphate resulted in successive increases in

RBC ATP concentrations.[9] In 1993, they described acceptable in vivo RBC recoveries after 8 and 9 weeks of storage in 200 mL of an ammonium- and phosphate-containing AS, but they stated that the ammonium would have to be removed at the end of storage.[10] In 1990, Mazor et al.[11] showed that the ammonium and potassium in Meryman Solution 6 were not critical, as they could be replaced with sodium or rubidium, a monovalent cation about the same size as ammonium, with no difference in the initial rise in RBC ATP concentrations. In 1994, Mazor et al.[12] showed that the RBC ATP concentration was most directly affected by the pH and the adenine and phosphate content. In 1992, Kay and Beutler[13] examined the mechanism by which Meryman's Solution 6 increased stored RBC ATP concentrations. They concluded that the inhibition of phosphofructokinase by ATP was blocked by the ammonia in the solutions, which led to higher than normal concentrations of ATP. Also in 1992, Dumaswala and colleagues[14] compared SAGM solutions supplemented with glutamine or glutamine and phosphate. The phosphate-containing solution showed increased RBC ATP concentrations. Finally, in 1993, Högman and collaborators[15] produced a storage solution, in which half-strength citrate in the anticoagulant and a mixture of monosodium and disodium phosphate in the AS led to a higher pH and phosphate content in the final storage solution. When used as a 100-mL AS, this solution allows RBC storage for 7 weeks, and it is now licensed in Europe as ErythroSol (Baxter International, Deerfield, IL).

The data from these studies did not directly address the effects of storage solution volume, but they suggested that it might be possible to make directly infusable, 200-mL, 8- or 9-week RBC storage solutions. Therefore, we undertook to measure directly the effect of AS volume by using two experimental and two conventional RBC storage solutions. In Study 1, increasing volumes of AS-1 resulted in lower RBC ATP concentrations but also in less hemolysis in a volume-dependent manner. In Study 2, increasing volumes of EAS-61 resulted in increased RBC ATP concentrations and reduced hemolysis in a volume-dependent manner. In Study 3, AS-5, an AS-1-like SAGM solution, and EAS-61 were directly compared along with a third 300-mL EAS, EAS-64. Storage in the EASs again resulted in greater RBC ATP concentrations and lower hemolysis during storage from those with conventional volumes of the licensed solution.

The increased phosphate concentration and pH of the EASs appears to lead to increased RBC ATP synthesis and thus to higher RBC ATP concentrations throughout storage. Högman et al.[15] demonstrated this effect in a 7-week storage solution. Increasing the AS volume raised the final concentration of phosphate and adenine in the storage bag and appeared to drive the RBC ATP concentrations even higher. The increased storage solution volume also had a beneficial effect, improving RBC morphology and reducing storage hemolysis. This effect of increased AS volume has not previously been recognized. The effect is important, for if EAS-61 were used as an AS at a 100-mL volume, it would not meet the current FDA storage solution licensing requirement of less than 1 percent hemolysis during storage beyond about 7 weeks.[16] Moreover, the improvement in storage hemolysis observed with increasing volumes of EAS-61 solution appears to be greater than that observed with AS-1. This suggests that the reduced hemolysis is an effect of both the increased storage volume and the better maintenance of RBC energy metabolism. Hypotonic swelling of the cells does not appear to be critical for this volume-related reduction in hemolysis, because the beneficial effect was observed with storage in increasing volumes of isotonic AS-1. Thus, it is not obvious from the data why storage solution volume is important. The cells, although mixed weekly for sampling, spend most of their time at very high local concentrations in the bottom of the storage bag.

EAS-61 and EAS-64 are directly infusable RBC storage solutions composed of saline, adenine, dextrose, mannitol, and disodium phosphate in acceptable concentrations. These materials are already used in approved RBC storage solutions in the United States and/or Europe. The downside of pooling studies, such as the ones described here, is that, after the blood is pooled, it cannot be returned to the original donor in studies of RBC recovery and survival. Only the surrogates of recovery—RBC ATP concentration and RBC morphology—can be measured in such studies. However, the data presented here do suggest that EAS-61 and EAS-64 should be clinically tested to determine the adequacy of 24-hour in vivo RBC recovery after storage for 7, 8, 9, and 10 weeks. Longer-lasting and better RBC storage will reduce RBC losses, improve the availability of blood in remote locations, make autologous transfusion programs more practical, and improve transfusion safety.

REEFERENCES

1. Hess JR. Extended liquid storage of red blood cells. In: Manning FJ, Sparacino L, eds. Blood donors and the supply of blood and blood products. Washington: National Academy Press, 1996:99-102.
2. Vengelen-Tyler V, ed. Technical manual. 12th ed. Bethesda: American Association of Blood Banks, 1996.
3. Solheim BG, Bergerud UE, Kjeldsen-Kragh J, et al. Improved blood preservation with 0.5CPD erythro-sol. Coagulation factor VIII activity and erythrocyte quality after delayed separation of blood. Vox Sang 1998;74:168-75.
4. Walker WH, Netz M, Ganshirt KH. 49 day storage of erythrocyte concentrates in blood bags with the PAGGS-mannitol solution. Beitr Infusionsther 1990;26:55-9.
5. Meryman HT, Hornblower MLS, Syring RL. Prolonged storage of red cells at 4°C. Transfusion 1986;26:500-5.
6. Moore GL, Ledford ME, Merydith A. A micromodification of the Drabkin Hb assay for measuring plasma Hb in the range of 5 to 2000 mg/dl. Biochem Med 1981;26:167-73.

7. Usry RT, Moore GL, Manalo FW. Morphology of stored, rejuvenated human erythrocytes. Vox Sang 1975;28:176-83.
8. Greenwalt TJ, McGuinness CG, Dumaswala UJ, Carter HW. Studies in red blood cell preservation. 3. A phosphate-ammonium-adenine AS. Vox Sang 1990;58:94-9.
9. Dumaswala UJ, Oreskovic RT, Petrosky TL, Greenwalt TJ. Studies in red blood cell preservation. 5. Determining the limiting concentrations of NH_4Cl and Na_2HPO_4 needed to maintain red blood cell ATP during storage. Vox Sang 1992;62:136-40.
10. Greenwalt TJ, Dumaswala UJ, Dhingra N, et al. Studies in red blood cell preservation. 7. In vivo and in vitro studies with a modified phosphate-ammonium AS. Vox Sang 1993;65:87-94.
11. Mazor D, Dvilansky A, Meyerstein N. Prolonged storage of red cells with ammonium chloride and mannitol. Transfusion 1990;30:150-3.
12. Mazor D, Dvilansky A, Meyerstein N. Prolonged storage of red cells: the effect of pH, adenine and phosphate. Vox Sang 1994;66:264-9.
13. Kay A, Beutler E. The effect of ammonium, phosphate, potassium, and hypotonicity on stored red cells. Transfusion 1992;32:37-41.
14. Dumaswala UJ, Wilson MJ, José T, Daleke DL. Glutamine- and phosphate-containing hypotonic storage media better maintain erythrocyte membrane physical properties. Blood 1996;88:697-704.
15. Högman CF, Eriksson L, Gong J, et al. Half-strength citrate CPD combined with a new AS for improved storage of red blood cells suitable for clinical use. Vox Sang 1993;65:271-8.
16. Davey RJ. Red cell radiolabeling in transfusion medicine. In: Davey RJ, Wallace ME, eds. Diagnostic and investigational uses of radiolabeled blood elements. Arlington: American Association of Blood Banks, 1987:39-66.

Appendix 5 (Pages 94-114)

Title: The Effects of Polyvinyl Chloride and Polyolefin Blood Bags on Red Blood Cells Stored in a New Additive Solution.

Authors: Hill HR, Oliver CK, Lippert LE, and Hess JR.

THE EFFECTS OF POLYVINYL CHLORIDE AND POLYOLEFIN BLOOD BAGS ON RED BLOOD CELLS STORED IN A NEW ADDITIVE SOLUTION

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Running Head: Effects of PVC blood bags

Abstract:

Background: Packed red blood cells (PRBCs) must be stored in polyvinyl chloride (PVC) bags plasticized with di-2-ethylhexyl phthalate to achieve their full storage life with conventional storage solutions. Better additive solutions may remove this requirement.

Study Design and Methods: Two hundred mL of Experimental Additive Solution - 61 (EAS-61) maintains PRBCs for 9 weeks. Twenty four units of PRBCs were pooled in groups of 4 units, each pool was realiquoted into 4 units, and stored, six pooled units per arm, in 1) 100 mL of EAS-61 in PVC, 2) 200 mL of EAS-61 in PVC, 3) 100 ml of EAS-61 in polyolefin (PO), and 4) 200 mL of EAS-61 in PO. Hemolysis, RBC morphology indices, RBC ATP concentrations, and other measures of RBC metabolism and function were measured weekly.

Results: RBC hemolysis exceeded 1% by 3 and 5 weeks in PO bags containing 100 and 200 mL of EAS-61. In PVC bags, hemolysis reached 1% at 8 and 10 weeks. RBC ATP concentrations were 1 μmol/g Hb higher at 2 weeks in the PVC-stored units.

Conclusions: RBCs stored in PVC had markedly less hemolysis and higher RBC ATP concentrations than those stored in PO. Hemolysis would limit PRBC storage to 4 weeks in PO bags.

Key Words: Blood storage, RBC storage, Humans, Plastics, Polyvinyl chloride, Polyolefin, RBC Hemolysis, RBC ATP concentrations, Di-2-ethylhexyl phthalate

Introduction:

Large activities, such as health care, by their very nature create secondary health risks for patients, employees, and the general public. Attempting to understand the risks and benefits of routine health care activities, such as blood transfusion, can lead to better methods which reduce risk and to better understanding of what risks are necessary to achieve desired benefits. Plastic blood bags are an example of a technical advance that makes transfusion safer for patients, makes blood handling safer for health care workers, and reduces the weight burden of medical waste on the environment.[1] However, plasticizers leach into patients,[2] bagmakers are exposed to carcinogenic plastic monomers,[3] and the ozone layer is degraded by incineration of medical wastes containing halogenated hydrocarbons.[4]

Polyvinyl chloride (PVC) plasticized with di-2-ethylhexyl phthalate (DEHP) is the standard material used for red blood cell storage bags. The risks of PVC are well documented,[3] while the risks of DEHP are more questionable;[5] but the benefit of this plastic material for blood bankers is a prolongation of the acceptable storage time of packed RBCs from 3 to 6 weeks.[6]

Recently, we have developed a storage solution, EAS-61, which can effectively store packed RBCs for 9 weeks in PVC.[7] To evaluate whether this improved storage solution might work as well in a different plastic, we conducted a study comparing storage of PRBCs in PVC with storage in polyolefin (PO). We pooled 24 units of blood in groups of 4 units then realiquoted and stored them in 1) 100 mL of EAS-61 in PVC, 2) 200 mL of EAS-61 in PVC, 3) 100 ml of EAS-61 in polyolefin (PO), and 4) 200 mL of EAS-61 in PO. Blood stored in PO hemolysed more rapidly then in PVC, and the benefits of storing blood for 4 weeks in PO versus 9 weeks in PVC did not seem favorable.

Materials and Methods:

Volunteers

Twenty-four healthy volunteers, who were donating full units of blood for the collection of anthrax-immune plasma, consented to their RBCs being used for storage studies. The donors met standard blood donor criteria and gave informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the U.S. Army's Human Use Research Review Board. Volunteers were tested to exclude sickle hemoglobin trait (Sicklescreen®, Pacific Hemostasis, Huntersville, NC).

Storage Solution

The EAS-61 solution (Composition: NaCl 26 mM, adenine 2 mM, glucose 50 mM, mannitol 20 mM, Na_2HPO_4 12 mM) was prepared in the laboratory from high purity adenine, sugars, salts and water and sterilely filtered into one-liter polyolefin storage bags (code 4R2238, Baxter Healthcare Corp., Roundlake, IL). The bags were held at 37°C for two weeks. On the eighth day, a sample was sent to a commercial reference laboratory (The Associates of Cape Cod, Falmouth, MA) for endotoxin testing using the Limulus Amoebocyte Lysate assay. At the end of two weeks, the solutions were cultured. The cultures were incubated for another two weeks. Sterility was confirmed by the absence of bacterial growth in the cultures after 14 days.

Study Design

We conducted a "pooling" study to evaluate RBC morphology, metabolism, and integrity over the course of 11 weeks of storage in PVC and PO bags. Pooling reduces the largest source

of variability in conventional blood storage studies, the differences between the RBCs from different donors,[7] by placing some of the cells from every donor in every arm of the study while maintaining conventional unit size. Twenty-four RBC units, unreactive on the indirect antiglobulin test (IAT), were grouped into sets of 4 ABO-matched units. Each set was then pooled, mixed, and aliquoted to make four identical pooled units. Blood was handled in a way intended to minimize contact with PVC plastic until the pooled PRBCs were aliquoted.

After mixing the four units in each pooled set of PRBCs with either 100 or 200 mL of EAS-61 additive solution in either PVC or PO bags, the units were stored at 1-6°C for 11 weeks. Electrolytes, pH, metabolites, blood gases, RBC morphology indices, hemolysis, and RBC adenosine-5'-triphosphate (ATP) concentrations were measured weekly.

RBC Unit Preparation

Standard units (450 ± 45 ML) of blood were collected from each donor into 63 mL of CPD anticoagulant in one-liter PO bags (4R2238, Baxter healthcare Corp., Roundlake, IL). The bag had been modified into a collection bag by sterile transfer of the CPD and the needle with a short length of the PVC tubing from a commercial triple bag collection set (BB*AGD456A, Terumo Medical Corp, Elkton MD) using a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton MD). Twenty of the units were drawn on a mobile blood drive and were placed in the refrigerator at 4°C for 1 to 6 hours before transport in a shipping container with wet ice 40 miles to the laboratory. Four units were drawn in the laboratory and processed within 4 hours. Packed cells were prepared by centrifugation at 5,000 x g for 5 minutes at room temperature followed by the removal of sufficient plasma to achieve a hematocrit of 85%. ABO/Rh and IAT

testing were performed using tube methods and commercial reagents (Immucor, Inc., Norcross, GA). ABO-matched, IAT-non-reactive PRBCs were then pooled in groups of four in separate, empty one-liter PO bags, mixed thoroughly, and aliquoted into the study units by weight using sterile tubing connection for all transfers. The aliquots were transferred to either 600 mL PVC transfer bags (code 4R2023, Baxter Healthcare Corp., Roundlake, IL) or one-liter PO storage bags (code 4R2238, Baxter Healthcare Corp, Roundlake, IL). Either 100 or 200 mL of EAS-61 was added to the PRBC aliqout prior to storage.

In vitro measurements

Samples from units were collected, after gentle mixing by inversion, by a sterile sampling procedure in a biological safety cabinet. A battery of *in vitro* tests was performed on all units at the beginning of storage and weekly thereafter.

The total hemoglobin (Hb) concentration, WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically using the modified Drabkin assay.[8] Percent hemolysis was determined by the ratio of free to total hemoglobin. The results are expressed as percent hemolysis to compensate for the differences in hematocrit and Hgb concentrations between samples. Centrifuged microhematocrits (Clay Adams, Becton-Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzers.

RBC ATP, glucose, and lactate concentrations were measured in supernatants of deproteinized PRBCs. Packed cell aliquots were mixed with cold 10% trichloroacetic acid to

precipitate blood proteins, centrifuged at 2700 x g for 10 minutes, and the protein free supernatant frozen at -80°C until tested. ATP was assayed enzymatically using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Supernatant chloride, pH, and blood gases were measured on a blood gas analyzer (Corning 855, Ithaca, NY). Thus, pH was measured at 37°C. Sodium, potasium, phosphate, lactate and glucose were measured on a programmable chemical analyzer (Hitachi 902 Analyzer, Boehringer Mannheim Corporation, Indianapolis, IN). The average degree of RBC shape change from discocytes to echinocytes to spherocytes was measured according to the method of Usry, Moore, and Manalo.[9]

Statistical Analysis

Comparisons of means of measured values at given times between the arms of the trial were evaluated by analysis of variance. Probabilities less likely than 0.05 were considered statistically significant.

Results:

Significant differences were observed between the PVC- and PO-stored cells in RBC ATP concentrations, RBC morphology, and fractional hemolysis. Figure 1A shows that in the early phases of storage, RBC ATP concentrations averaged 1 μmol/g Hb higher in the PVC-stored cells. This is the result of a more rapid increase in RBC ATP concentration in the first two weeks of storage. At the end of storage this difference diminished as the RBC ATP concentration decreased more slowly in the PO-stored cells. This difference was not related to differences in the

glucose consumption as decreasing glucose concentrations (data not shown) and increasing lactate concentrations (Figure 1B) were the same for equivalent volumes of EAS-61. RBC morphology indices were also lower in PO-stored cells after the first week of storage (Figure 1C). The rate of decrease in the indices was twice as great in PO compared to PVC. There was a small effect of increased storage volume leading to slightly better morphology. Finally, RBC stored in PO had 4 times the hemolysis of cells stored in PVC (Figure 1D). In each plastic, doubling the EAS-61 volume reduced the hemolysis by half. RBC hemolysis exceeded 1% by 3 and 5 weeks in PO bags containing 100 and 200 mL of EAS-61. The measured values were 0.89 ± 0.10 % at 2 weeks and 1.33 ± 0.22 % at 3 weeks in 100 mL in PO and 0.99 ± 0.30 % at 4 weeks and 1.24 ± 0.32 % at 5 weeks in 200 mL in PO. In PVC bags, hemolysis reached 1% at 8 and 10 weeks in the 100 and 200 mL volumes respectively. Again, the measured values were 0.96 ± 0.19 % at 7 weeks and 1.17 ± 0.59 % at 8 weeks in 100 mL in PVC and 0.93 ± 0.39 % at 9 weeks and 1.09 ± 0.53 % at 10 weeks in 200 mL in PVC.

Gas transport was also different in the two kinds of bags, but it did not effect storage solution pH (Figure 2A). The partial pressure of CO_2 (Figure 2B) and the concentration of bicarbonate (Figure 2C) were higher in the PVC bag after the first week. The oxygen partial pressure in the PO bags was higher at all time points after the initial measurement (Figure 2D). The shoulder of the oxygen saturation curve was reached at 3 weeks in PO and 10 weeks in PVC.

Electrolytes were not effected by storage bag composition. Supernatant sodium concentration decreased (Figure 3A) and potassium concentration increased (Figure 3B) with storage. The chloride concentration of the suspending fluid (Figure 3C) increased for the first two weeks and then slowly decreased. Phosphate concentration in the suspending solution

(Figure 3D) decreased in the first two weeks of storage and slowly increased thereafter. The sum of the concentrations of the cations, sodium and potassium, and the anions, chloride, bicarbonate, phosphate, and lactate, were approximately equal when corrected for valence.

Discussion:

This study was performed to determine if an improved RBC storage solution would eliminate the requirement for PVC blood bags to achieve 6 week or longer RBC storage. The results showed that despite achieving high concentrations of RBC ATP and low fractional hemolysis when RBCs were stored in PVC, when the same cohort of cells was stored in PO they had lower RBC ATP concentrations and four times greater hemolysis at each weekly measurement. Thus, RBCs that have a clinically acceptable hemolyzed fraction of less than 1 % after 7 weeks in 100 mL of EAS-61 or 9 weeks in 200 mL when stored in PVC bags, have greater than 1% hemolysis at 3 weeks or 5 weeks when stored in PO bags in equivalent volumes.

The increased hemolysis may be related to the more rapid morphologic changes that RBCs undergo in the course of storage in PO bags. RBCs stored in conventional storage solutions change shape from discocytes to echinocytes to spherocytes over time. Exposure of warm blood to PO bags for just a few hours in the course of pooling caused a rapid initial shape change which persisted even after the RBCs were transferred to PVC bags. We have previously described RBCs stored continuously in PVC bags in 100 or 200 mL of EAS-61[7]. These cells had a more discoid morphology throughout storage, yet very similar rates of hemolysis with time.

It is also not clear why the RBC ATP concentrations are higher in the PVC-stored cells. The rates of glucose consumption and lactate formation were the same, as were the extracellular

pH and phosphate concentrations. In the first two weeks of storage when the differences in ATP concentrations appeared, the RBC morphology indices were all almost identical. RBC ATP was either made less efficiently, or broken down by a pathway that did not effect cell shape or electrolyte concentration.

The PO bags have greater gas permeability than the PVC bags, carbon dioxide pressures were lower and oxygen pressures were higher in the PO bags during most of the storage interval. However, the bicarbonate concentrations, which might effect differences in intracellular pH were not greatly different, especially in the first two weeks. The higher oxygen pressures in the PO bags might be expected to contribute to oxidative RBC membrane damage. However, the rate of formation of oxygen free radicals by the conversion of hemoglobin to methemoglobin, their major source in stored blood, is highest at partial hemoglobin saturations of 50-75% or about 40 mmHg of oxygen partial pressure.[10] The oxygen partial pressures remain in this range longer in the PVC bags. It should be noted that all the measured partial pressures are artificially high because the units were stored at 4°C but measured at 37°C on a blood gas machine. The partial pressures of the gases increase as their solubility decreases with increasing temperature.

PVC blood bags plasticized with DEHP improve conventional RBC storage by reducing hemolysis, microvesiculation, and the membrane loss that leads to increased osmotic fragility.[11] [Greenwalt TG, McGuinness CG, Dumaswala UJ. Studies in red blood cell preservation: 4. Plasma hemoglobin exceeds free hemoglobin. Vox Sang 1991; 61:14-17.] The increased storage time that these materials allow makes blood banking more efficient by reducing outdating. The potential benefits of using an improved storage solution such as EAS-61 in PVC bags with a 9 week shelf-life might include reduced outdating, the ability to maintain larger blood supplies at

remote locations, and more practical systems of autologous storage. The solution may also be safer because the reduced hemolysis and improved RBC morphology are associated with fewer RBC breakdown products in the bag. These are advantages that blood bankers desire for their institutions and for their patients.

Any potential environmental advantage of reducing the production of ozone depleting halogenated hydrocarbons by reducing the incineration of PVC blood bags is probably lost by the creation of inefficiencies associated with the loss of RBC shelf-life in the blood system. More donors would have to travel to donate the additional blood required to make up for the increased outdating. More specimens would have to be shipped to regional and national testing laboratories. More waste would ultimately be created, leading to greater pressure for the incineration of all medical waste, and likely to burning of more non-blood-bag PVC. It seems unlikely that shortening the shelf-life of blood would lead to improvements in overall environmental quality.

Nevertheless, research to reduce the environmental impact of blood bank activities is important. As in this study, it can shed light on the cellular mechanisms important in blood storage. It forces blood bankers to think clearly about the risk inherent in all the activities we perform. It can lead to safer, more effective, and more cost efficient blood systems and a healthier world for all.

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The authors certify that they are employed, directly or indirectly, by the U.S. Army which has filed for patent rights on the EAS-61 experimental additive solution. One of the authors, JRH, is listed as an inventor on the patent filing. This research was supported by the Combat Casualty Care Program of the U.S. Army Medical Research and Materiel Command

Bibliography:

1. Knudtson KP. Plastic blood containers. Amer J Clin Path 1953; 23:813-7.

2. Jaeger RJ, Rubin RJ. Migration of a phthalate ester plasticizer from polyvinyl chloride blood bags into stored human blood and its location in human tissues. N Eng J Med 1972; 287:1114-8.

3. Lewis R. Vinyl chloride and polyvinyl chloride. Occup Med 1999: 14:719-24.

4. Montzka SA, Butler JH, Myers RC, et al. Decline in the tropospheric abundance of halogen from halocarbons: Implications for stratospheric ozone depletion. Science 1996: 272:1318-22.

5. Turnbull D, Rodricks JV. Assessment of possible carcinogenic risk to humans resulting from exposure to di(2-ethylhexyl)phthalate (DEHP). J Amer College Tox 1985; 4:111-39.

6. Myhre BA, Johnson D, Marcus CS. Survival of red cells stored for 21 and 35 days in a non-di-(2-ethylhexyl)phthalate plastic container. Vox Sang 1987: 53:199-202.

7. Hess JR, Lippert LE, Derse-Anthony CP, et al. The effects of phosphate, pH and additive solution volume on RBCs stored in saline-adenine-glucose-mannitol additive solutions. Transfusion (in press, ms99172).

8. Moore GL, Ledford ME, and Merydith A. A micromodification of the Drabkin hemoglobin assay for measuring plasma hemoglobin in the range of 5 to 2000 mg/dl. Biochem Med 1981; 26:167-73.

9. Usry RT, Moore GL, Manalo FW. Morphology of stored, rejuvenated human erythrocytes. Vox Sang 1975; 28:176-83.

10. Rifking JM, Zhang L, Levy A, Manoharan PT. The hypoxic stress on erythrocytes associated with superoxide formation. Free Radic Res Commun 1991; 12-13:645-52.

11. Greenwalt TG, McGuinness CG, Dumaswala UJ. Studies in red blood cell preservation: 4. Plasma hemoglobin exceeds free hemoglobin. Vox Sang 1991; 61:14-17.

Figure Legends

Figure 1. Weekly measures of A) RBC ATP concentration, B) RBC lactate concentration, C) RBC morphologic index, and D) fractional RBC hemolysis for the RBCs stored in 1) 100 mL of EAS-61 in PVC (--◊--), 2) 200 mL of EAS-61 in PVC (--◆--), 3) 100 ml of EAS-61 in PO (--○--), and 4) 200 mL of EAS-61 in PO (--●--). RBC ATP concentrations were higher in the early phases of storage in PVC bags, but the amount of lactate produced did not differ. RBCs stored in PO bags showed greater morphologic change and hemolysis. Data presented as mean ± standard error of the mean (n=6 pooled units per group).

Figure 2. Weekly measures of A) supernatant pH, B) CO_2 partial pressure, C) bicarbonate concentration, and D) oxygen partial pressure for the RBCs stored in 1) 100 mL of EAS-61 in PVC (--◊--), 2) 200 mL of EAS-61 in PVC (--◆--), 3) 100 ml of EAS-61 in PO (--○--), and 4) 200 mL of EAS-61 in PO (--●--). Diffusion of CO_2 out and oxygen into the PO bags was about 3 times more rapid than observed with PVC bags, but there was no significant effect on pH. Data presented as mean ± standard error of the mean (n=6 pooled units per group).

Figure 3. Weekly measures of A) supernatant sodium concentration, B) supernatant potassium concentration, C) supernatant chloride concentration, and D) supernatant inorganic phosphate concentration for the RBCs stored in 1) 100 mL of EAS-61 in PVC (--◊--), 2) 200 mL of EAS-61 in PVC (--◆--), 3) 100 ml of EAS-61 in PO (--○--), and 4) 200 mL of EAS-61 in PO (--●--). EAS-61 volume but not bag composition effected electrolyte composition. Data presented as

mean ± standard error of the mean (n=6 pooled units per group).

Figure 1.

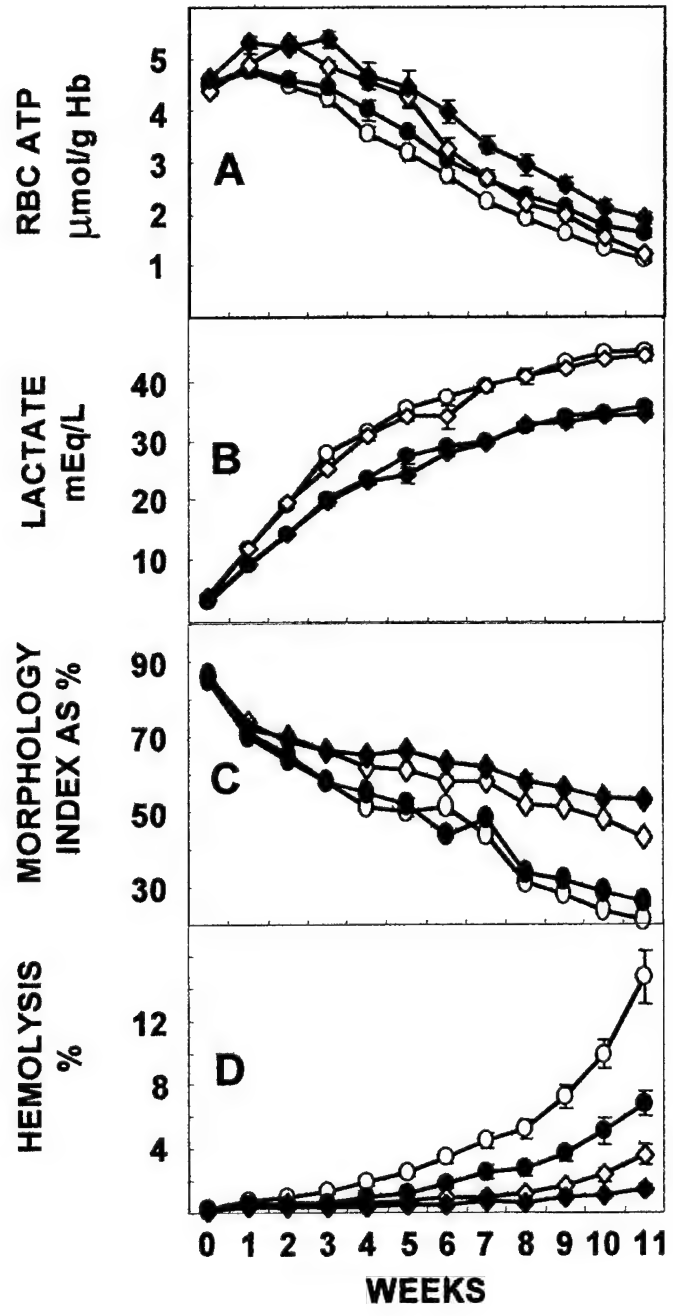
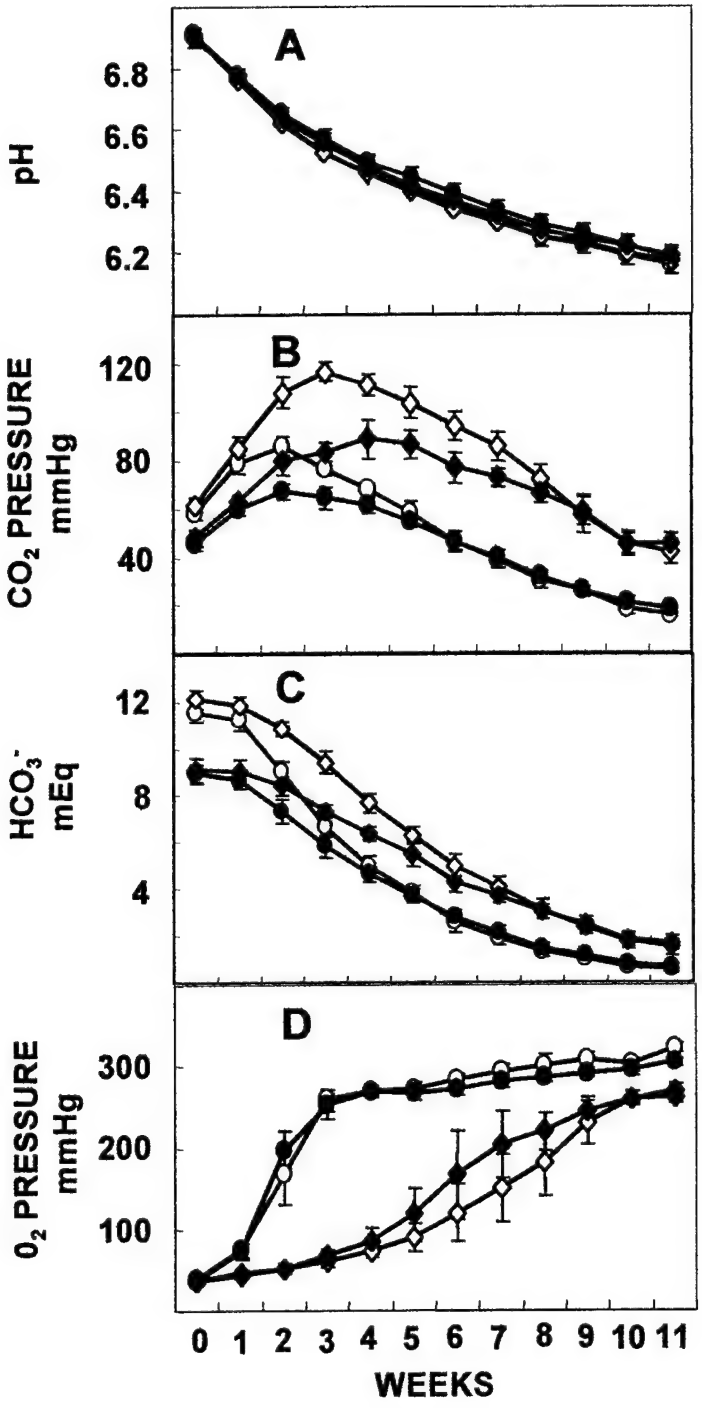

RBC ATP µmol/g Hb
5
4
3
2
1
A
LACTATE mEq/L
40
30
20
10
B
MORPHOLOGY INDEX AS %
90
70
50
30
C
HEMOLYSIS %
12
8
4
D
0 1 2 3 4 5 6 7 8 9 10 11
WEEKS

Figure 2.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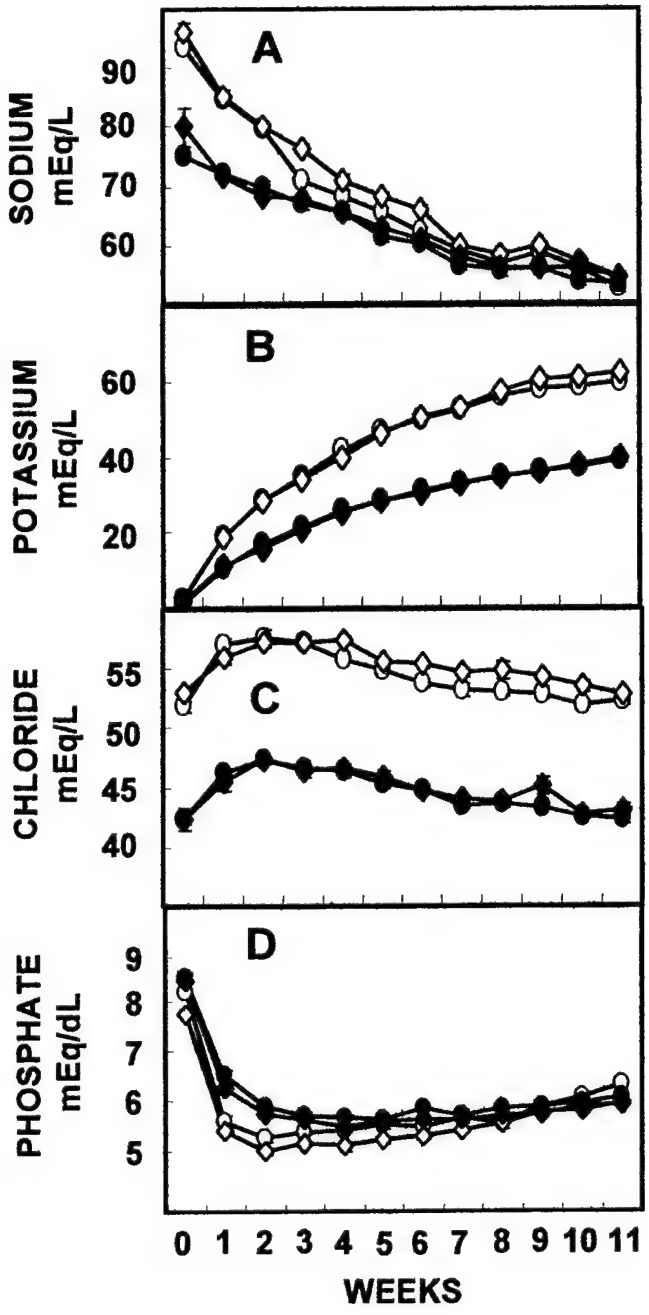
pH
6.8
6.6
6.4
6.2
B
CO_2 PRESSURE mmHg
120
80
40
C
HCO_3^- mEq
12
8
4
D
0_2 PRESSURE mmHg
300
200
100
0 1 2 3 4 5 6 7 8 9 10 11
WEEKS

Figure 3.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80
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60
B
POTASSIUM
mEq/L
60
40
20
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mEq/L
55
50
45
40
D
PHOSPHATE
mEq/dL
9
8
7
6
5
0 1 2 3 4 5 6 7 8 9 10 11
WEEKS

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The authors represent that they have contributed substantively to the development of the content of this paper including the (1) concept or design of the work; (2) analysis or interpretation of the data; (3) drafting or revising the article for content; and (4) approval of the version to be published. The authors believe the manuscript represents valid work and take public responsibility for it.

The authors certify that they are employed, directly or indirectly, by the U.S. Army which has filed for patent rights on the EAS-61 experimental additive solution. One of the authors, JRH is listed as an inventor on the patent filing. Project support from the U.S. Army is identified on the title page of the manuscript.

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Appendix 6 (Pages 116-120)

Data tables and graphs for *in vitro* protocols **"Comparison of EAS-61 and AS-3 RBC Stored in either 100 mL or 200mL of Additive Solution: Liquid Storage"** and **"Comparison of EAS-61 and AS-3 RBC Stored in either 100 mL or 200 mL of Additive Solution: Frozen Storage"**.

Figure legend:

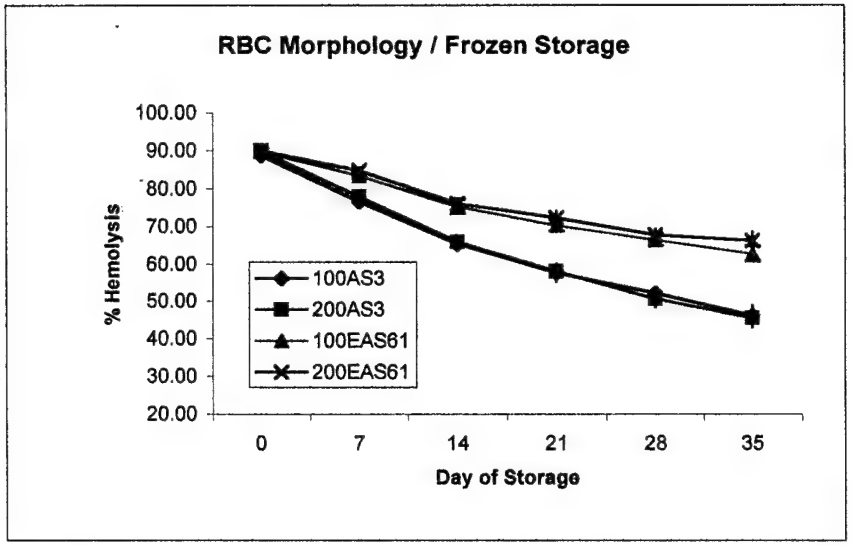
Diamond = 100 mL AS-3 Storage Solution

Square = 200 mL AS-3 Storage Solution

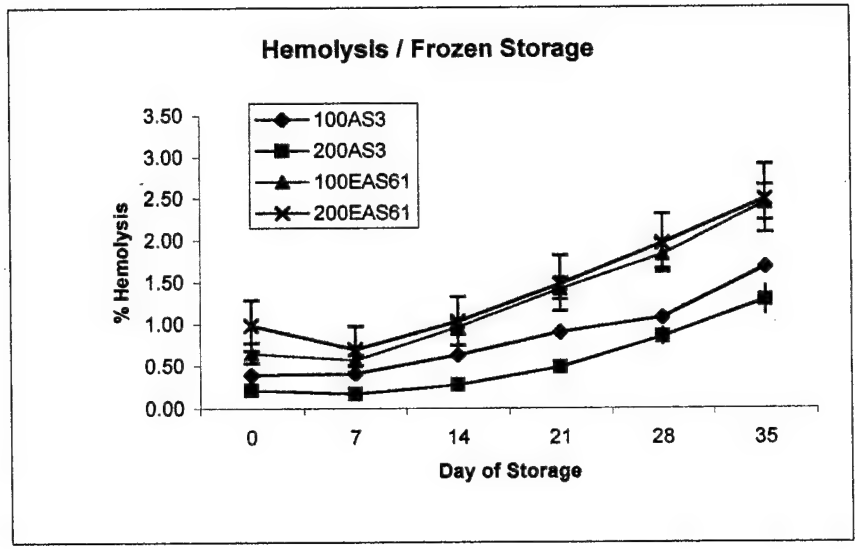
Triangle = 100 mL EAS-61 Storage Solution

"X" = 200 mL EAS-61 Storage Solution

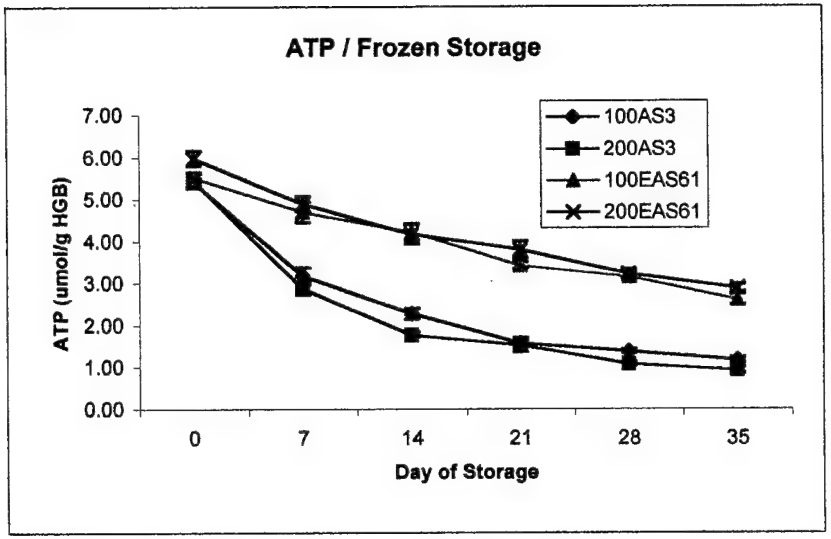
Day	AS3 - 100mL	AS3 - 200mL	EAS61 - 100mL	EAS61 - 200mL
0	88.80	90.00	89.80	90.00
7	76.70	78.00	83.50	84.80
14	65.50	66.00	75.30	76.20
21	57.70	58.00	70.30	72.30
28	52.20	50.70	66.50	67.80
35	46.30	45.50	62.80	66.30
Standard errors of the mean				
0	1	0.54	0.91	0.95
7	1.36	0.82	0.68	0.73
14	1.97	1.5	1.91	1.04
21	2.27	1.91	1.5	1.72
28	1.32	2.13	1.63	1.32
35	2.45	2.38	1.97	2.04



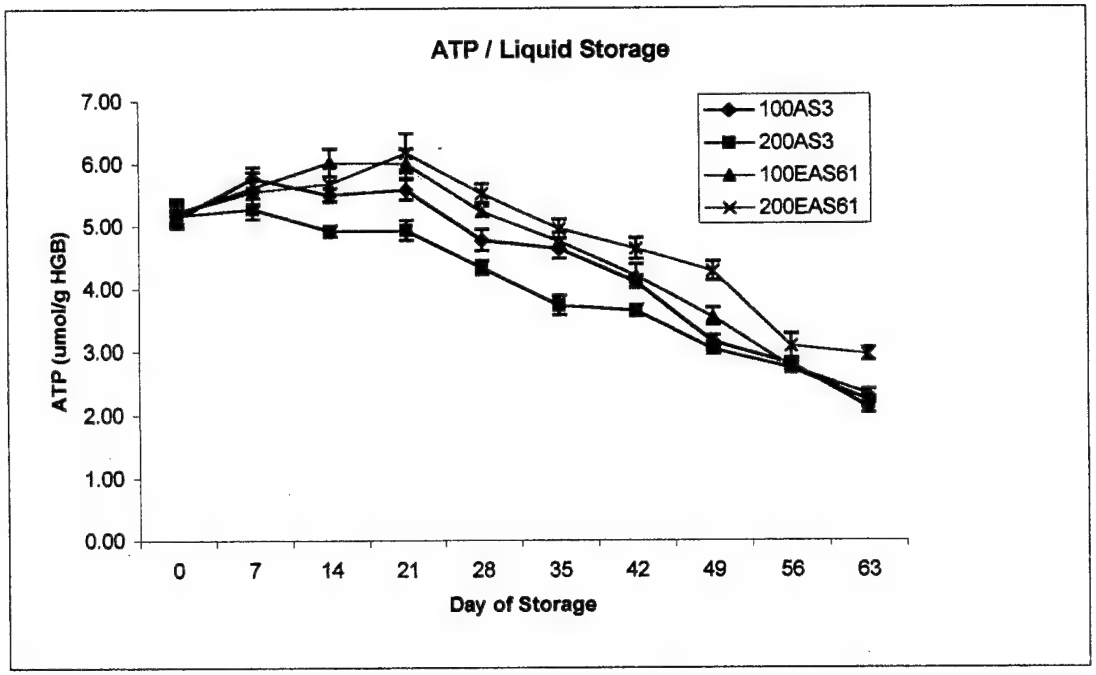
Day	AS3 - 100mL	AS3 - 200mL	EAS61 - 100mL	EAS61 - 200mL
0	0.40	0.22	0.65	0.98
7	0.41	0.17	0.57	0.70
14	0.63	0.28	0.96	1.03
21	0.90	0.49	1.42	1.48
28	1.07	0.85	1.83	1.96
35	1.68	1.28	2.44	2.49
Standard errors of the mean				
0	0.06	0.04	0.12	0.3
7	0.05	0.01	0.06	0.27
14	0.01	0.02	0.09	0.29
21	0.04	0.03	0.13	0.33
28	0.08	0.09	0.17	0.35
35	0.06	0.17	0.21	0.41



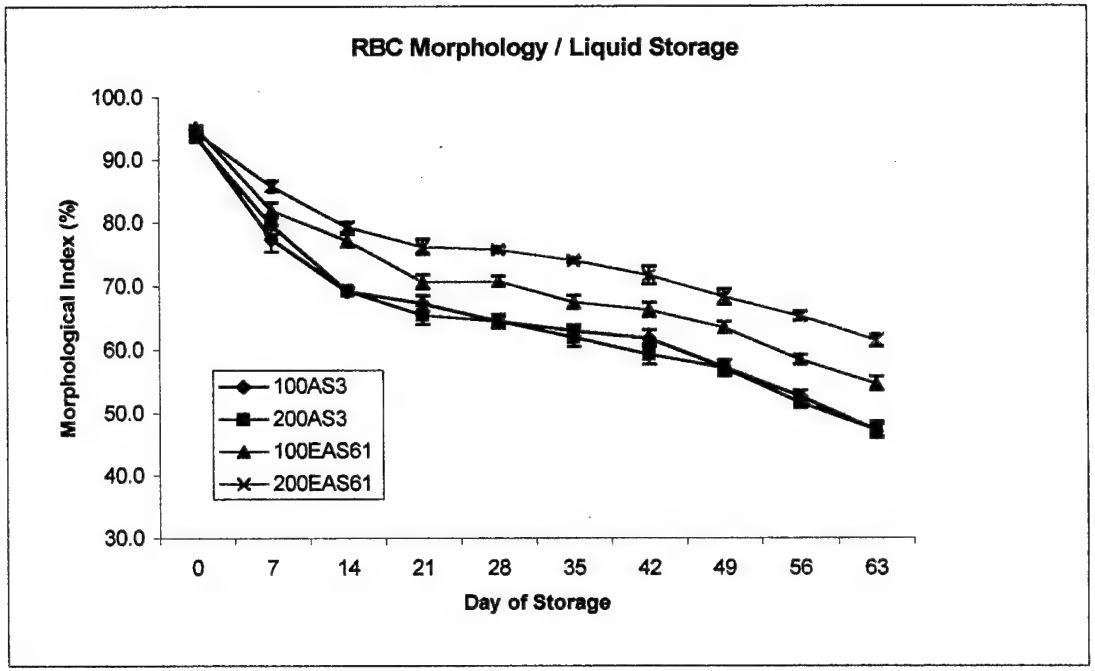
Day	AS3 - 100mL	AS3 - 200mL	EAS61 - 100mL	EAS61 - 200mL
0	5.42	5.45	5.53	5.98
7	3.18	2.88	4.70	4.90
14	2.28	1.78	4.22	4.17
21	1.57	1.53	3.43	3.80
28	1.38	1.08	3.15	3.23
35	1.17	0.93	2.62	2.88
Standard errors of the mean				
0	0.08	0.17	0.12	0.18
7	0.2	0.14	0.24	0.18
14	0.13	0.05	0.2	0.23
21	0.08	0.17	0.08	0.19
28	0.04	0.05	0.13	0.11
35	0.06	0.07	0.13	0.09



Day	100AS3	200AS3	100EAS61	200EAS61
0	5.13	5.17	5.22	5.22
7	5.77	5.28	5.62	5.55
14	5.50	4.92	6.02	5.68
21	5.58	4.93	6.00	6.17
28	4.77	4.33	5.23	5.52
35	4.63	3.73	4.75	4.95
42	4.10	3.65	4.20	4.63
49	3.15	3.03	3.55	4.27
56	2.8	2.73	2.75	3.08
63	2.12	2.23	2.33	2.95
Standard error of the mean				
0	0.16	0.18	0.17	0.22
7	0.18	0.17	0.25	0.21
14	0.11	0.09	0.22	0.28
21	0.16	0.16	0.23	0.31
28	0.17	0.11	0.09	0.15
35	0.16	0.15	0.14	0.15
42	0.1	0.09	0.18	0.17
49	0.1	0.08	0.14	0.15
56	0.07	0.06	0.08	0.19
63	0.09	0.05	0.07	0.1



Day	100AS3	200AS3	100EAS61	200EAS61
0	94.0	93.7	95.2	94.5
7	77.5	79.7	82.0	85.8
14	69.2	69.3	77.2	79.3
21	67.3	65.5	70.7	76.2
28	64.5	64.5	70.8	75.7
35	63.0	62.0	67.5	74.0
42	61.8	59.3	66.3	71.7
49	57.3	57.2	63.5	68.3
56	52.7	51.7	58.5	65.3
63	47.2	47.3	54.7	61.5
Standard error of the mean				
0	0.41	0.41	0.34	0.27
7	2.11	1.5	1.22	0.88
14	0.48	0.5	1.02	0.82
21	1.18	1.5	1.09	1.16
28	0.68	1.09	0.77	0.54
35	0.95	1.5	1.02	0.41
42	1.32	1.59	1.09	1.41
49	1.09	1.25	0.88	1.18
56	0.86	0.91	0.75	0.68
63	1.02	1.32	1.13	0.95



Appendix 7 (Pages 122-128)

Data tables and graphs for *in vitro* protocols **"Eleven Week Red Blood Cell Storage in 300 mL of Lower Salt Variants of Bicarbonate Containing EAS-67"**

Short Name: Low Salt

Figure legend:

Diamond = 300 mL EAS-67 Storage Solution

Square = 300 mL EAS-W1 Storage Solution

Triangle = 300 mL EAS-W2 Storage Solution

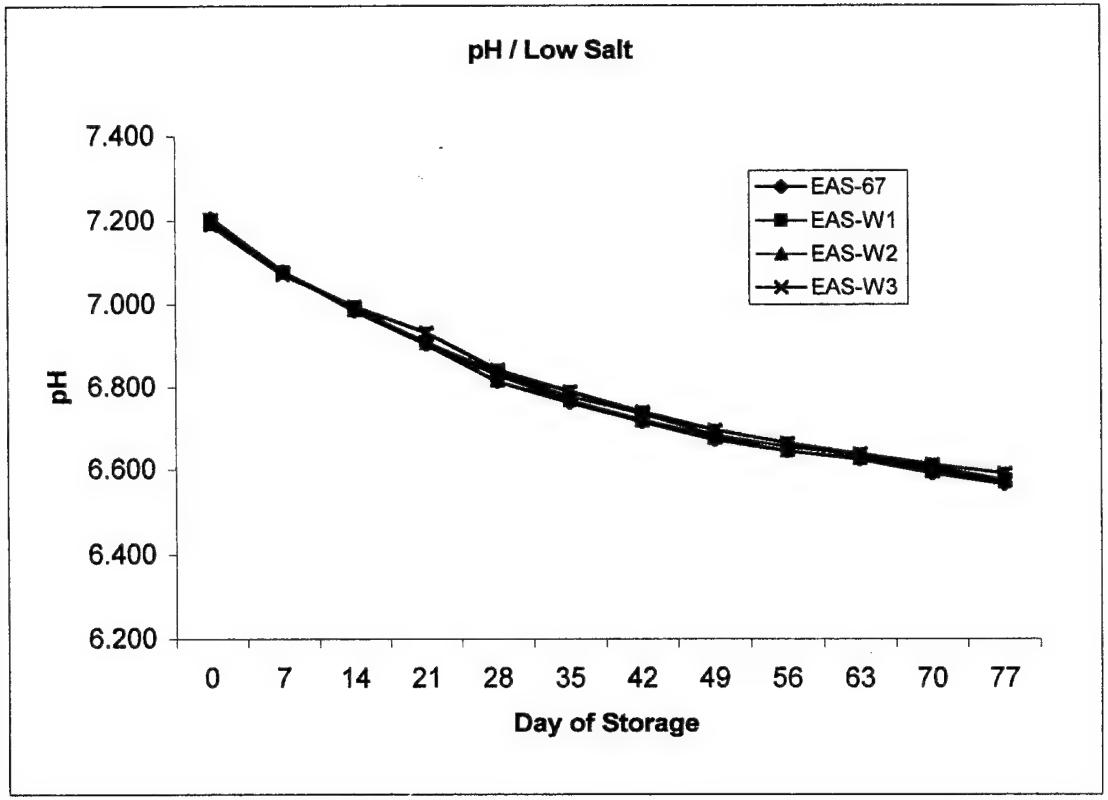
"X" = 300 mL EAS-W3 Storage Solution

pH

Day	EAS-67	EAS-W1	EAS-W2	EAS-W3
0	7.207	7.201	7.194	7.191
7	7.080	7.078	7.076	7.072
14	6.984	6.994	6.990	6.995
21	6.903	6.904	6.909	6.931
28	6.813	6.830	6.835	6.842
35	6.761	6.766	6.776	6.789
42	6.715	6.720	6.734	6.739
49	6.672	6.677	6.683	6.695
56	6.644	6.644	6.654	6.664
63	6.624	6.628	6.635	6.638
70	6.592	6.601	6.608	6.613
77	6.566	6.571	6.577	6.591

Standard error of the mean

0	0.01	0.01	0.01	0.01
7	0.01	0.01	0.01	0.01
14	0.01	0.01	0.01	0.00
21	0.01	0.01	0.01	0.01
28	0.01	0.01	0.01	0.01
35	0.00	0.01	0.01	0.01
42	0.01	0.00	0.01	0.01
49	0.01	0.01	0.01	0.01
56	0.01	0.01	0.01	0.01
63	0.01	0.01	0.00	0.01
70	0.01	0.00	0.01	0.01
77	0.00	0.01	0.01	0.01

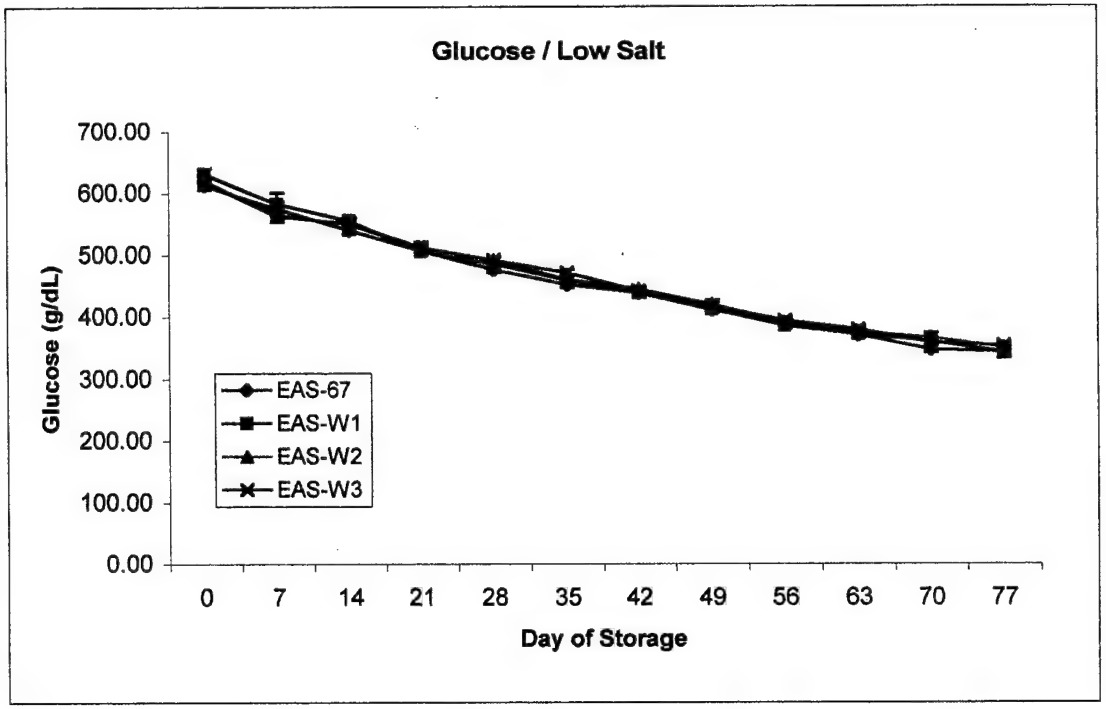


Glucose

Day	EAS-67	EAS-W1	EAS-W2	EAS-W3
0	613.55	631.95	621.00	621.00
7	575.20	583.50	566.15	562.90
14	540.25	555.35	549.15	551.40
21	506.85	506.42	511.55	512.05
28	476.16	485.35	491.45	491.55
35	451.95	458.90	461.20	470.95
42	439.25	440.00	444.50	441.70
49	411.60	412.45	420.65	417.55
56	388.65	386.35	390.80	394.20
63	370.60	372.35	375.95	380.00
70	347.95	360.50	366.75	358.28
77	343.00	343.30	350.65	353.20

Standard error of the mean

0	7.12	9.33	6.33	4.45
7	11.98	17.6	9.62	8.82
14	6.39	5.29	6.27	5.96
21	6.86	6.57	6.42	9
28	5.04	4.27	5.74	4.86
35	4.49	7.87	5.06	5.99
42	8.14	5.88	3.35	6.33
49	4.45	5.74	6.56	5.7
56	3.96	5.61	4.84	5.1
63	5.06	3.37	3.01	4.63
70	6.02	5.85	6.76	4.33
77	9.32	4.86	7.74	5.69

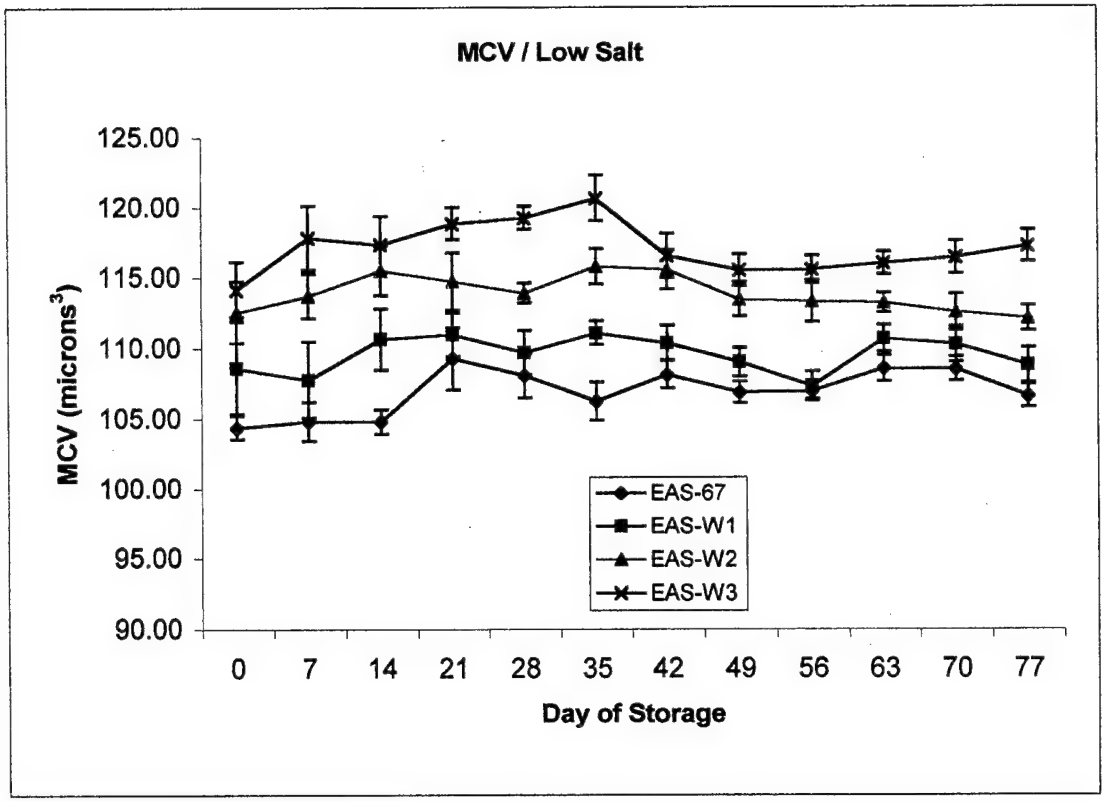


MCV (from Hematology Analyzer)

Day	EAS-67	EAS-W1	EAS-W2	EAS-W3
0	104.37	108.64	112.56	114.19
7	104.82	107.79	113.72	117.84
14	104.81	110.67	115.51	117.36
21	109.27	110.97	114.74	118.84
28	108.07	109.69	113.92	119.24
35	106.23	111.09	115.79	120.63
42	108.14	110.37	115.55	116.55
49	106.88	109.00	113.45	115.51
56	106.97	107.34	113.30	115.56
63	108.57	110.68	113.21	116.00
70	108.53	110.28	112.54	116.40
77	106.59	108.78	112.10	117.23

Standard error of the mean

0	0.83	3.29	2.17	1.95
7	1.39	2.69	1.57	2.28
14	0.87	2.17	1.74	2
21	2.23	1.56	2.02	1.14
28	1.59	1.57	0.71	0.84
35	1.36	0.83	1.25	1.63
42	0.98	1.23	1.4	1.57
49	0.75	1.01	1.21	1.11
56	0.61	1.03	1.48	0.98
63	0.92	0.92	0.7	0.79
70	0.86	1.24	1.29	1.15
77	0.81	1.23	0.9	1.12

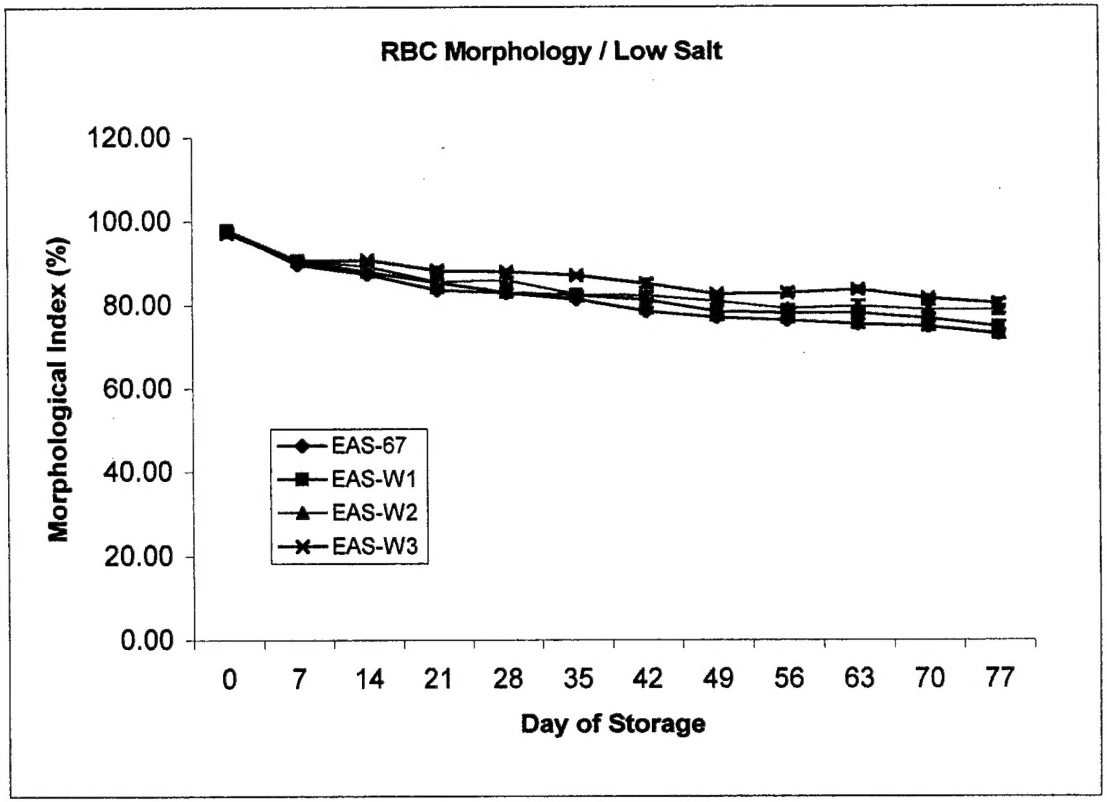


RBC Morphology

Day	EAS-67	EAS-W1	EAS-W2	EAS-W3
0	97.80	97.80	97.50	97.20
7	89.80	90.70	90.70	90.70
14	87.30	88.00	89.30	90.80
21	83.50	85.30	85.50	88.30
28	82.80	83.00	86.00	88.00
35	81.30	82.30	82.50	87.00
42	78.50	81.20	82.20	85.00
49	77.00	78.50	80.80	82.50
56	76.20	78.00	79.00	82.80
63	75.30	78.00	79.70	83.50
70	74.70	76.70	78.80	81.50
77	72.80	74.70	78.80	80.20

Standard error of the mean

0	0.48	0.34	0.48	0.5
7	0.48	0.5	0.68	0.5
14	0.64	0.54	0.41	0.36
21	0.54	0.59	0.54	0.73
28	0.91	0.68	0.54	0.41
35	0.41	0.82	0.54	0.54
42	0.68	0.5	0.75	0.95
49	0.82	1.22	0.92	0.41
56	0.59	0.68	0.82	0.88
63	1.04	0.68	1.13	0.68
70	1.09	0.82	1.04	0.61
77	1.02	1.22	0.91	1.00

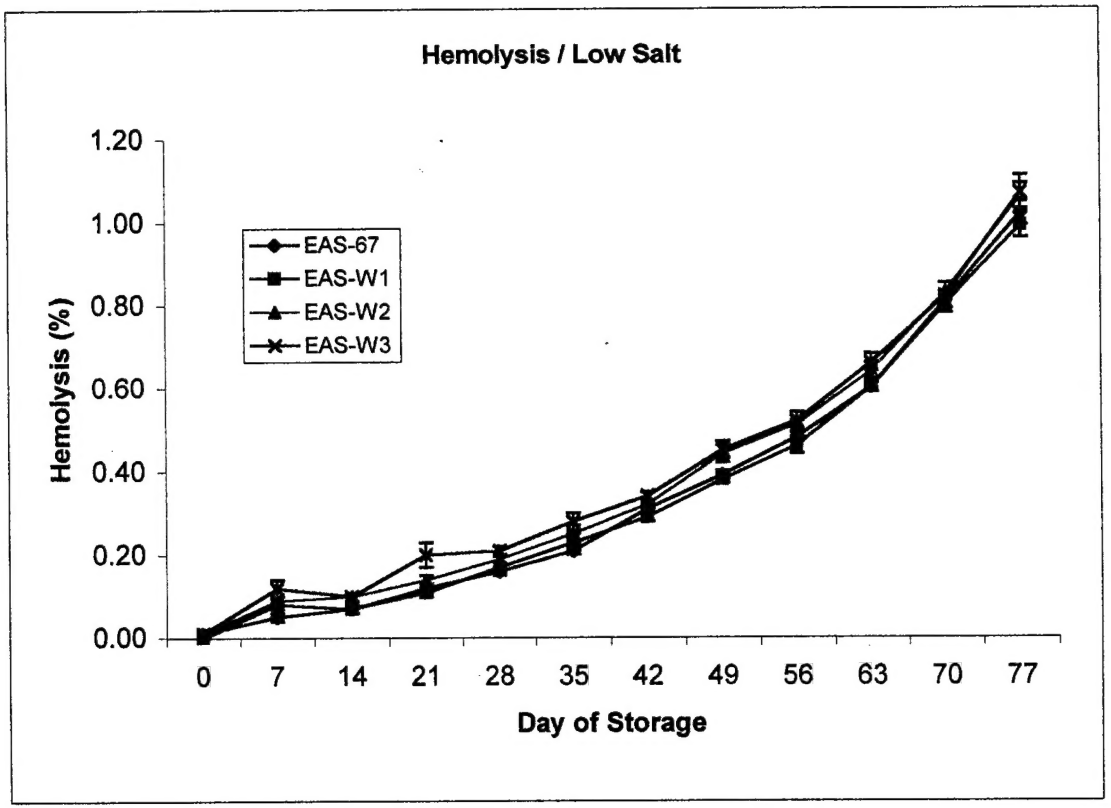


Hemolysis

Day	EAS-67	EAS-W1	EAS-W2	EAS-W3
0	0.01	0.00	0.01	0.01
7	0.05	0.08	0.09	0.12
14	0.07	0.07	0.10	0.10
21	0.12	0.11	0.14	0.20
28	0.16	0.17	0.19	0.21
35	0.21	0.23	0.25	0.28
42	0.31	0.29	0.32	0.34
49	0.39	0.38	0.44	0.45
56	0.48	0.46	0.51	0.52
63	0.60	0.60	0.64	0.66
70	0.81	0.80	0.83	0.82
77	1.02	0.99	1.06	1.07

Standard error of the mean

0	0	0	0.01	0.01
7	0.01	0.01	0.01	0.02
14	0.01	0.01	0.01	0.01
21	0.01	0.01	0.01	0.03
28	0.01	0.01	0.01	0.01
35	0.01	0.02	0.02	0.02
42	0.01	0.01	0.01	0.01
49	0.01	0.01	0.02	0.02
56	0.01	0.02	0.01	0.02
63	0.01	0.01	0.02	0.02
70	0.02	0.02	0.02	0.03
77	0.03	0.03	0.03	0.04

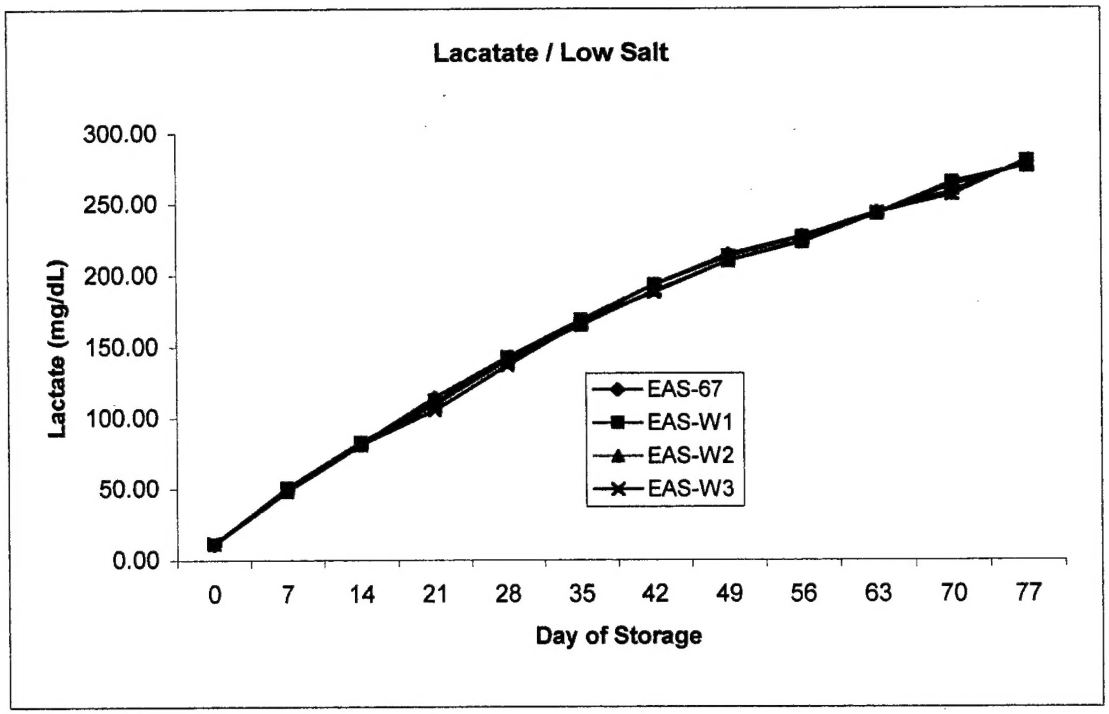


Lactate

Day	EAS-67	EAS-W1	EAS-W2	EAS-W3
0	11.20	11.60	11.50	11.90
7	50.20	50.80	48.40	48.75
14	81.70	83.30	81.10	81.20
21	114.60	110.50	109.90	105.65
28	142.90	142.70	140.60	137.25
35	168.20	169.10	165.10	167.20
42	193.20	193.50	189.20	188.00
49	214.10	212.80	209.90	209.55
56	227.00	226.40	222.70	223.65
63	244.00	243.00	242.60	243.20
70	258.40	265.10	262.80	256.20
77	279.50	277.00	276.40	280.35

Standard error of the mean

0	0.72	0.73	0.68	0.68
7	1.25	1.76	1.22	1.29
14	2.07	2	2.03	1.92
21	1.76	1.29	1.61	1.29
28	1.81	1.86	1.92	1.74
35	1.65	2.57	2.8	4.03
42	2.43	2.82	2.71	2.4
49	1.69	2.35	1.59	2.25
56	2.08	1.94	1.61	1.39
63	1.91	2.52	3.14	2.42
70	2.41	2.69	1.73	3.51
77	4.67	4.97	3.37	2.08



ATP

Day	EAS-67	EAS-W1	EAS-W2	EAS-W3
0	4.96	4.92	4.84	5.03
7	4.50	4.22	4.35	4.03
14	4.75	4.60	4.70	4.32
21	5.27	5.02	5.17	4.82
28	5.18	4.98	5.08	5.05
35	4.80	4.73	4.62	4.80
42	4.82	4.30	4.17	4.27
49	4.38	4.08	4.15	4.17
56	3.93	4.09	3.95	4.25
63	3.92	4.07	4.02	3.97
70	3.93	3.97	3.80	3.90
77	3.52	3.40	3.32	3.30

Standard error of the mean

0	0.2	0.2	0.18	0.15
7	0.12	0.14	0.17	0.15
14	0.21	0.19	0.2	0.15
21	0.15	0.12	0.16	0.16
28	0.1	0.13	0.14	0.12
35	0.11	0.11	0.16	0.18
42	0.14	0.08	0.07	0.05
49	0.14	0.04	0.06	0.08
56	0.06	0.02	0.08	0.1
63	0.1	0.15	0.09	0.11
70	0.07	0.06	0.11	0.11
77	0.07	0.11	0.13	0.11